UNIVERSITY OF ADELAIDE LIBRARY 22/2/05

Studies on HIV-1 Virion Infectivity Factor

Feng Feng

M.Sc.

Infectious Diseases Laboratories

Institute of Medical and Veterinary Science

Adelaide

South Australia

School of Molecular & Biomedical Science

University of Adelaide

Adelaide

South Australia

****

A thesis submitted to the University of Adelaide in fulfilment of the requirements for the degree of Doctor of Philosophy

October, 2004

Contents

Abstract

Virion Infectivity Factor (Vif) protein of human immunodeficiency virus type 1 (HIV-1) is essential for the productive viral infection of primary human CD4 T lymphocytes and macrophages. Recently, it has been reported that Vif overcomes the HIV-inhibitory effects of the cellular factor CEM15/APOBEC3G, which has cytidine deaminase activity. Using the yeast two-hybrid system, our laboratory previously reported the identification of a Vif-interacting ring finger protein called Triad 3 (renamed Triad 3au in this study), from a human leukocyte cDNA library. The full-length cellular protein homologue of Triad 3 has been recently identified as the zinc finger protein inhibiting NF*k*B (ZIN). In this study, biological characteristics of Triad 3au/ZIN were investigated. Sequence analysis indicated that Triad 3au protein contains all 4 major ring-like motifs of ZIN. RNA was extracted from A3.01 cells and RT PCR was then performed using the ZIN gene specific primers. A single product of 1,464 bps was obtained and subsequently confirmed as the full-length coding region of ZIN by sequence analysis. GST fusion protein pull-down experiments confirmed that overexpressed ZIN binds to purified Vif *in vitro* suggesting a direct interaction between ZIN and Vif. Next, in studies of co-transfected human 293T cells, Triad 3au/ZIN and Vif were shown to interact using co-immunoprecipitation and confocal microscopy demonstrated co-localisation of Triad 3au and Vif in cytoplasm and membrane while co-localisation of ZIN and Vif in nuclei.

To test the biological relevance of the Vif-ZIN interaction, infectious HIV-1 NL4.3 virus stocks were produced in 293T cells expressing Flag tagged ZIN or Flag tag

alone from transfected plasmids. The virus stocks produced in the presence of exogenously expressed ZIN were less infectious in both single-cycle infectivity assay and end-point titration compared with virus produced in the absence of exogenous ZIN. Real Time PCR analyses showed that cells infected with HIV NL4.3 virus stocks produced in the presence of exogenously expressed ZIN, showed reduced levels of early viral DNA synthesis. This reduction in viral reverse transcription and the reduction in single-cycle viral infectivity were shown to be dependent on the presence of Vif in the virus producer cells. The possible mechanisms by which presence of ZIN in producer cells reduces HIV-1 reverse transcription and replication in target cells are discussed.

Acknowledgments

This work was supported by the International Postgraduate Research Scholarship (IPRS) and Adelaide University Scholarship. This research was in part supported by a grant from the Australia Commonwealth AIDS Research Grant Programme.

Many thanks go to Dr. Peng Li and Prof. Chris Burrell for giving me the opportunity to pursue my Ph.D program in Australia and their invaluable guidance throughout the course of this Ph.D.

Dr. Adam Davis, many thanks for your patience and time with my project and correcting my paper and thesis. To Dr. Julie Lake, thank you very much for your teaching at the early stages of my Ph.D study.

To Dr. Jill Carr, Dr. Raman Sharma and Dr. Nick Vandegraaff, thank you for your guidance. You were always available whenever I had trouble with my project over the past years.

A great thank to Jennifer Clarke, Jane Arthur and Ghafer Sarvestani, for your technical guidance and useful discussions with my immunofluorescence experiments.

A special thankyou to Adrian Purins and Kelly Cheney for your jokes, laughs, all questions raised during my laboratory talks and other supports. These are highly appreciated.

To the entire HIV lab, both present (Carl Coolen, John Karlis, Satiya Wati, Sarah Martin, Branka Bauk) and past (Linda Mundy, Helen Hocking, Luke Dimasi, Robyn Taylor) for their helpful discussions and good friendships. Also Dr. Tuckweng Kok, thank you for your help with reviewing the figures and figure legends of my thesis.

To my family and relatives, thank you all very much for your understanding and utmost support during my studies.

Declaration of Originality

Real Time PCR analysis experiment described in Chapter 4 was done in collaboration with Dr. Adam J. Davis and Kelly Cheney. All DNA sequencing was performed by Mr. Arthur Mangos in the Institute of Medical and Veterinary Science. The rest of the work presented in this thesis was done by myself unless otherwise stated.

This thesis contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution.

To the best of my knowledge, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Feng FENG

Abbreviations

<	less than
>	greater than
°C	degrees Celcius
AIDS	Acquired Immune Deficiency Syndrome
bp	base pair
Ci	curie
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DDW	double distilled water
dGTP	2'-deoxynucleoside 5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	ethylene-diamine-tetra-acetic acid
g(unit)	gravity force
HIV-1	Human Immunodeficiency Virus Type 1
hr	hour(s)
IN	integrase
Kb	kilobase-pair
LB	Luria-Bertani medium
µg	microgram
µM	micromolar (micromoles per litre)

min	minute(s)
ml	millilitre
mM	millmolar (millimoles per litre)
ng	nanogram
OD	optical density
nt	nucleotide
^{32}P	radioactive phosphorous (mass number:32)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIC	preintegration complex
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
SDS	sodium dodecyl sulphate
s	second(s)
SSC	standard saline citrate
ssDNA	Strong-stop DNA
$TCID_{50}$	50% tissue culture infectious dose

Publications Related to this Study

1. Lake, J. A., Carr, J., Feng, F., Mundy, L., Burrell, C. & Li, P. (2003). The role of Vif during HIV-1 infection: interaction with novel host cellular factors. *Journal of Clinical Virology* **26**, 143-152.

2. Feng, F., Davis, A., Lake, J., Carr, J., Xia, W., Burrell, C & Li, P. (2004). Ring finger protein ZIN interacts with human immunodeficiency virus type 1 Vif. *Journal of Virology* **78**, 10574-10581

Chapter 1

Introduction

1. 1 Historical background of HIV/AIDS

Debate regarding the origin of AIDS has caused considerable interest and controversy since the beginning of the epidemic. There is now clear evidence that the human disease AIDS is caused by the virus termed human immunodeficiency virus (HIV), probably the descendant of an ancient simian immunodeficiency virus (SIV). SIV strains share close sequence homology with both HIV-1 and HIV-2. For example, HIV-2 is almost identical to a simian immunodeficiency virus found in the sooty mangabey monkey (SIVsm), also known as the green monkey, which is indigenous to western Africa (Hirsch et al., 1989).

A lentivirus was previously identified in two wild chimpanzees in Gabon, west equatorial Africa. This virus is referred to as SIV_{CPZ}. Sera from these animals cross-reacted with all the HIV-1 proteins including the envelope glycoproteins. Huet *et al* described the molecular cloning and sequencing of an infectious proviral clone of SIV_{CPZ}. The overall genetic organization was shown to be the same as that of HIV-1, but phylogenetic analysis revealed that the sequence was more divergent than any human HIV-1 sequence reported so far (Huet et al., 1990). Consistent research results were reported by other groups (Janssens et al., 1994, Vanden Haesevelde et al., 1996). Vanden Haesevelde isolated another HIV-1-related virus, SIV_{CPZ}-ANT, from a wild captured chimpanzee originating from Zaire (Vanden Haesevelde et al., 1996). In February 1999, researchers from the University of Alabama found that a

simian virus called SIVcpzUS isolated from frozen tissue of a chimpanzee common in central Africa was almost identical to human HIV-1 by sequence analyses (Gao et al., 1999). It was claimed that these chimpanzees were the source of HIV-1 and that the virus had, at some point, crossed species from chimpanzee to human. While this proposal is generally accepted, the precise details remain unclear.

Three of the earliest known instances of human HIV infection have been identified in samples dated from 1959 to 1976: 1. Antibody to HIV-1 was detected in a plasma sample taken in 1959 from an adult male living in what is now the Democratic Republic of Congo. 2. HIV was found in tissue samples from an African-American teenager who died in St. Louis in 1969. 3. Finally, HIV was found in tissue samples from a Norwegian sailor who died around 1976. These findings suggested that HIV was introduced into humans earlier, perhaps around the 1940s or the early 1950s. In fact computer modeling of HIV evolution by Dr. Bette Korber of the Los Alamos National Laboratory estimated that the first case of HIV infection probably occurred around 1930 in West Africa (Korber et al., 2000).

The first official case of AIDS was announced in the United States in 1981. Medical practitioners found a rare form of a relatively benign cancer called Kaposi's Sarcoma in several males. These patients also presented with pneumonia caused by *Pneumocystis carinii*, a condition normally only seen in individuals who were immunosuppressed. The Centers for Disease Control of the U.S. Public Health Service (CDC) named this new disease, Acquired Immune Deficiency Syndrome, or AIDS.

People afflicted with AIDS shared similar lifestyles including sexual behavior and intravenous drug use. These led researchers to suspect that the aetiological agent of AIDS was transmitted by body fluids, which was supported by the occurrence of AIDS cases amongst blood transfusion recipients. In 1983, Chermann and Barre-Sinoussi isolated a virus from the lymph node of patients with AIDS (Barre-Sinoussi et al., 1983, Chermann et al., 1983), which was subsequently termed Human Immunodeficiency Virus, or HIV. By 1984, an Enzyme Linked Immunosorbent Assay (ELISA) for the diagnosis of HIV infection was developed. By 1985, two independent groups reported the complete nucleotide sequence of HIV (Ratner et al., 1985, Wain-Hobson et al., 1985).

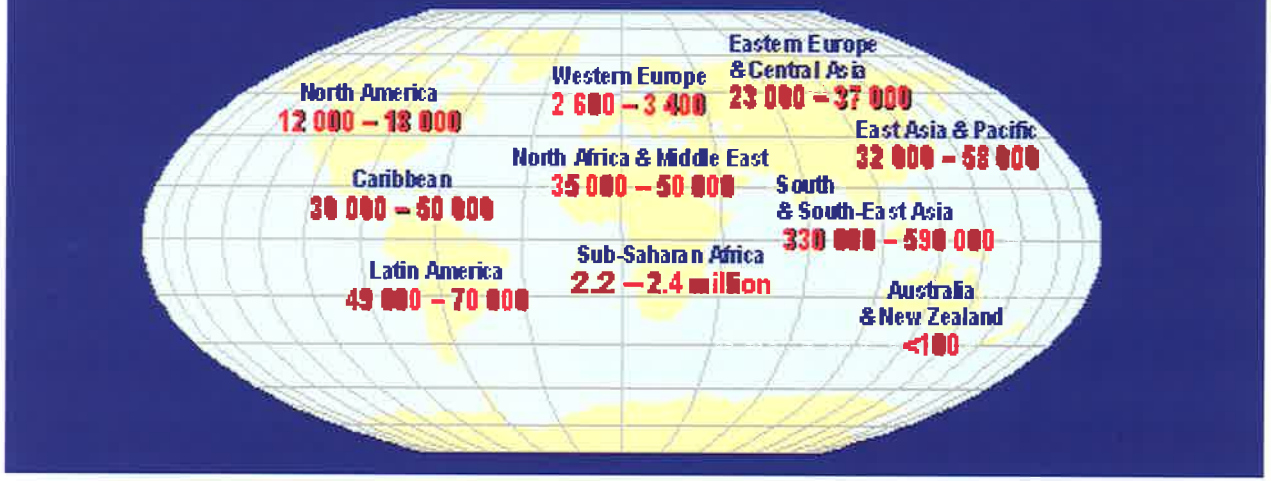
A number of factors have contributed to spread of the AIDS epidemic including use of unscreened blood transfusions, patterns of sexual behavior and intravenous drug use, of which the latter two continue to be a problem, particularly in less developed countries. According to December 2003 Figures from the Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization (WHO), the estimated number of adult and child deaths from HIV/AIDS during 2003 was 2.5-3.5 million (Figure1.1). Worldwide 4.2-5.8 million men, women and children were newly infected with HIV in 2003 (Figure1.2), that is, about 14,000 infections each day, and nearly 50 percent were among females. More than 95 percent of these new infections occurred in developing countries (UNAIDS. AIDS Epidemic Update, December, 2003).

Figure 1.1 Estimated number of deaths from HIV/AIDS in 2003

Global distribution and estimated numbers of adult and child deaths from HIV/AIDS in 2003 are presented. Numbers are estimated by UNAIDS and WHO

Adapted from http://www.unaids.org/EN/other/functionalities/Search.asp

Estimated adult and child deaths from HIV/AIDS during 2003



Total: 2.5 – 3.5 million

UNAIDS

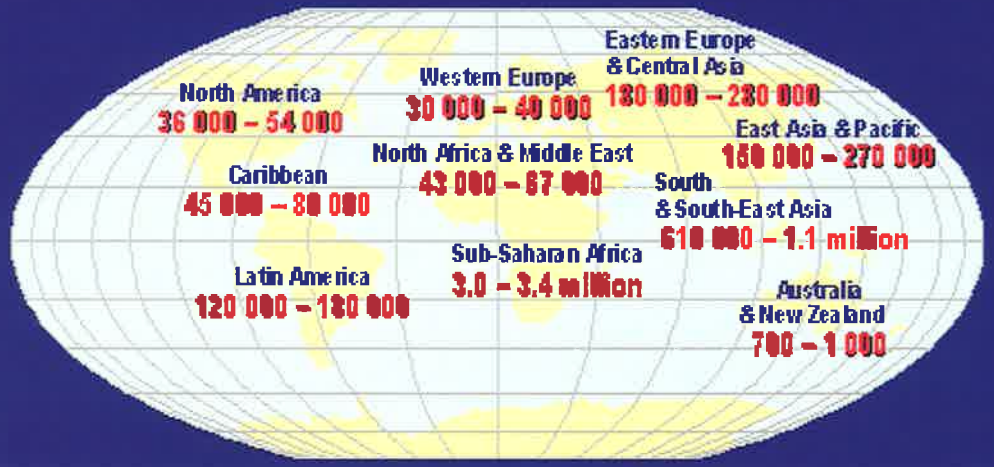
World Health Organization

Figure 1.2 Estimated number of new HIV infections in 2003

Global distribution and estimated numbers of adults and children newly infected with HIV in 2003 are presented. Numbers are estimated by UNAIDS and WHO

Adapted from http://www.unaids.org/EN/other/functionalities/Search.asp

Estimated number of adults and children newly infected with HIV during 2003



UNAIDS

Total: 4.2 – 5.8 million

World Health Organization

1.2 Overview of human immunodeficiency virus

1.2.1 Classification of HIV

Early electron microscope analysis of viral particles released from infected cell cultures revealed enveloped virions approximately 100 to 120 nm in size and of similar morphology to previously described retroviruses. The subsequent finding that HIV-1 possessed particle-associated reverse transcriptase further identified the virus as a member of the retrovirus family. The virus was assigned to the lentivirus genus on the basis of its distinctive corn shaped core, typical genome organisation including the presence of accessory proteins, and its chronic progressive course of infection.

Mature HIV-1 particles contain a cylindrical or conical core or nucleoid reminiscent of that previously described for visna virus (Gonda et al., 1985). Cloning and sequencing of proviral DNA purified from productively infected T-lymphocytes and T-cell leukemia cell lines, indicated that HIV-1 possessed a genomic organisation comparable with of the Retroviridae virus family (Ratner et al., 1985, Wain-Hobson et al., 1985). HIV-2 is a second sub-type of human immunodeficiency virus, which differs from HIV-1 in the nature of one of its accessory genes, namely *vpx* in HIV-2 and *vpu* in HIV-1 (Figure1.4). HIV-2 infection also tends to progress less rapidly, and the virus is more closely related to the simian immunodeficiency virus of sooty mangabeys (SIVsm). In 1992, Coffin proposed a new classification of retroviruses into seven genera based on nucleotide and amino acid sequence similarities as well as virion structures (Coffin, 1992). (see table 1.1)

Table 1.1

Classification of Retroviruses Proposed by Coffin in 1992

Genera	Type Species
Avian C-type	Avian leukosis virus, Rous sarcoma virus
Mammalian B-type	Mouse mammary tumour virus
Mammalian C-type	Moloney murine leukaemia virus, Spleen necrosis virus
Mammalian D-type	Mason-Pfizer monkey virus
Human T-cell Leukaemia/Lymphotropic Virus& Bovine leukaemia virus	HTLV & BLV
Spumavirus	Simian foamy virus
Lentivirus	Human immunodeficiency virus 1& 2, Visna virus, Equine infectious anaemia virus

Adapted from (Coffin et al, 1992)

1.2.2 HIV virion structure

Electron microscopy suggests that many viruses are roughly spherical. A detailed examination shows that they are actually icosahedral. HIV contains a lipid envelope surrounding the icosahedral protein shell comprising individual matrix protein (MA, p17), within which is a cone-shaped core composed of capsid protein (CA, p24) containing two identical copies of viral genomic RNA and the viral encoded protein, reverse transcriptase (RT). The viral RNA genome is a positive sense, single-stranded RNA molecule approximately 9,200 nucleotides long. The two molecules are non-covalently associated with each other within the virion to form a genomic RNA dimer. Also within the capsid are the viral encoded enzymes integrase (IN) and protease (PR), the viral protein Vpr, and other macromolecules derived from the cell including $tRNA^{lys3}$, which serves as a primer for the reverse transcription. Inserted in the virus envelope is the transmembrane glycoprotein (gp41), coupled noncovalently with a protruding surface envelope glycoprotein (gp120). The function of the envelope proteins is to enable HIV to attach and fuse with a target cell (Figure1.3).

1.2.3 Genetic organization of HIV

The genomes of prototypical retroviruses such as avian leukosis virus (ALV) and murine leukemia virus (MuLV) contain only three genes encoding the structural proteins *gag* (group-specific antigen), *pol* (polymerase) and *env* (envelope) proteins. In addition to *gag*, *env* and *pol* genes shared by all retrovirus, the HIV-1 genome encodes an additional six genes; *nef*, *rev*, *tat*, *vif*, *vpr* and *vpu,* which have accessory or regulatory functions (Figure1.4). The single-stranded (+) sense RNA genome is converted into double-stranded DNA by a process called reverse transcription (RTN). A short repeat sequence termed "R" forms a direct repeat at both ends of the

Figure 1.3 Structural model of the HIV-1 virion

Structure and composition of the HIV-1 virion indicated proposed location of viral proteins, RNA and components.

p24 (CA);	capsid protein
p17 (MA);	matrix protein
gp41;	transmembrane protein
gp120;	surface envelope protein
p32 (IN);	integrase
p10 (PR);	protease
p51/p66 (RT);	reverse transcriptase
MHC proteins;	major histocompatibility complex
ssRNA;	single-stranded RNA

Modified figure from http://tutor.lscf.ucsb.edu

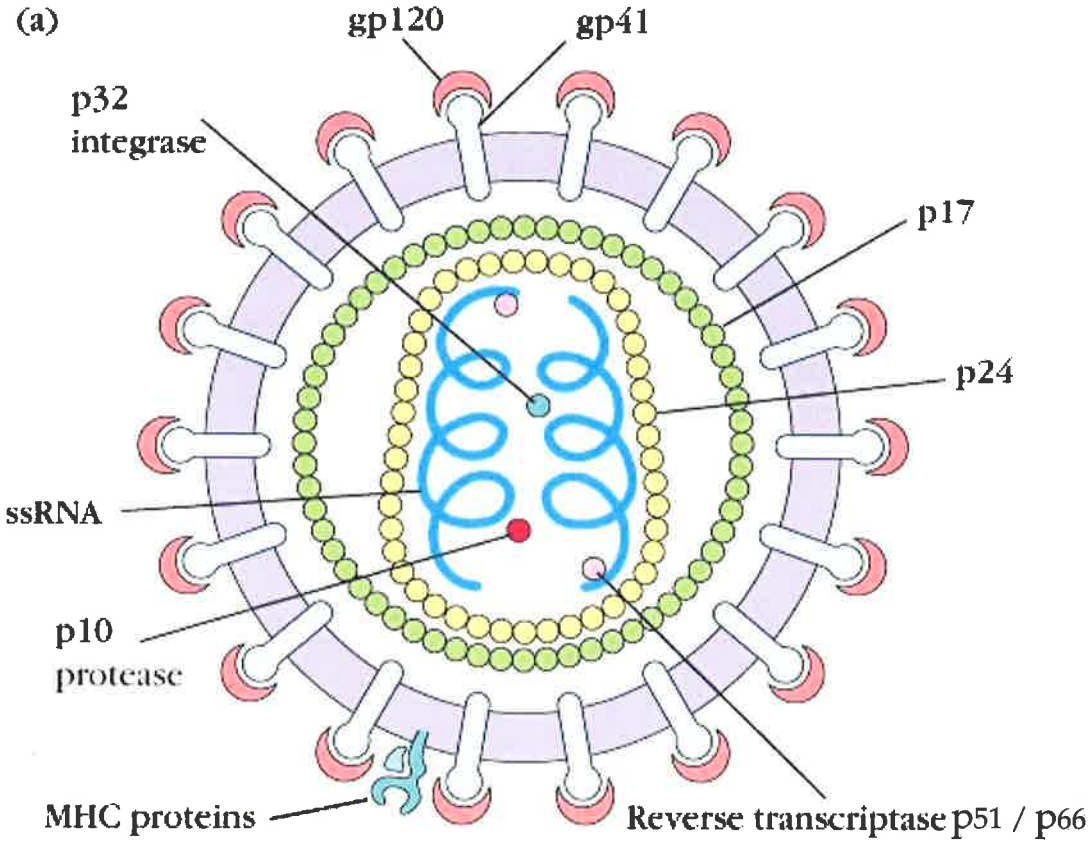

(a)
gp120
gp41
p32
integrase
p17
p24
ssRNA
p10
protease
MHC proteins
Reverse transcriptase p51 / p66

Figure 1.4 Organization of the HIV-1 genome

Relative size and position of viral genes are indicated using open boxes. Proviral genome contains 2 long terminal repeats (LTR) flanking the 5' and 3' termini of the viral RNA genome, represented by a continuous horizontal line.

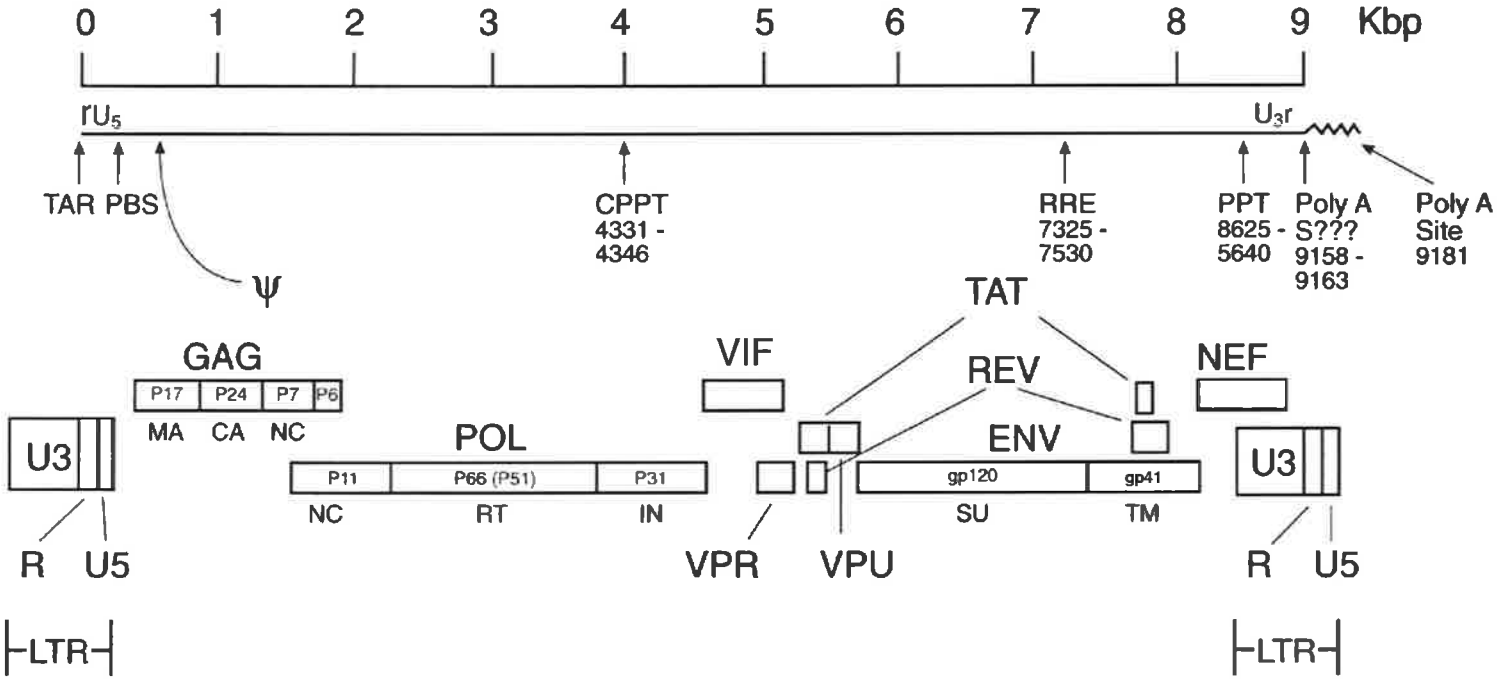
TAR: trans activating region

PBS: primer binding site

CPPT: central polypurine tract

RRE: Rev response element

PPT: polypurine tract


0
1
2
3
4
5
6
7
8
9
Kbp
rU5
U3r
TAR
PBS
Ψ
CPPT
4331 -
4346
RRE
7325 -
7530
PPT
8625 -
5640
Poly A
S???
9158 -
9163
Poly A
Site
9181
TAT
GAG
VIF
REV
NEF
P17
P24
P7
P6
MA
CA
NC
POL
ENV
U3
P11
P66 (P51)
P31
gp120
gp41
NC
RT
IN
SU
TM
U3
R
U5
VPR
VPU
R
U5
LTR
LTR

RNA genome. Inside this, U5 and U3 form unique non-coding regions located at the 5' and 3' ends of the genome respectively (Figure1.4). Located just downstream of U5 is a primer binding site (PBS) that is complementary to the 3' end of a specific tRNA primer used by the virus to initiate reverse transcription. There is a conserved polypurine tract (PPT) just upstream of U3, and in addition, the HIV-1 genome is unique amongst retroviruses in having a central PPT. The PPT RNA sequences are protected from degradation by the RNase H activity of RT, therefore providing a primer for initiating (+) strand viral DNA synthesis during reverse transcription (Figure1.4). The product of reverse transcription is a double-stranded DNA molecule slightly longer than the RNA genomic template in that it contains a second U3 at the 5' end and a second U5 at the 3' end. Thus each end of the molecule consists of U3RU5, a regulation sequence termed the long terminal repeat (LTR). (Figure1.4).

1.2.4 HIV replication cycle

HIV predominantly infects T lymphocytes and macrophages, which express CD4 on their surface. Surface viral protein gp120 binds the CD4 receptor resulting in virus attachment (Smith et al., 1987, Weiss et al., 1988). More recently, specific co-receptors were identified to be required for virus entry (Alkhatib et al., 1996, Choe et al., 1996, Wu et al., 1996). Following virus entry the RNA genome is reverse transcribed in cytoplasm of the host cell. Newly synthesised viral DNA then localizes to the host cell nucleus where it is integrated into the cell chromosome. Transcription of viral DNA into messenger RNA by cellular machinery results in an early and late stage of viral protein expression, regulated by viral proteins Tat and Rev. Final expression of late stage structural viral proteins results in assembly of new

progeny virus. Details of the stepwise HIV-1 replication cycle are illustrated in Figure1.5 and described below.

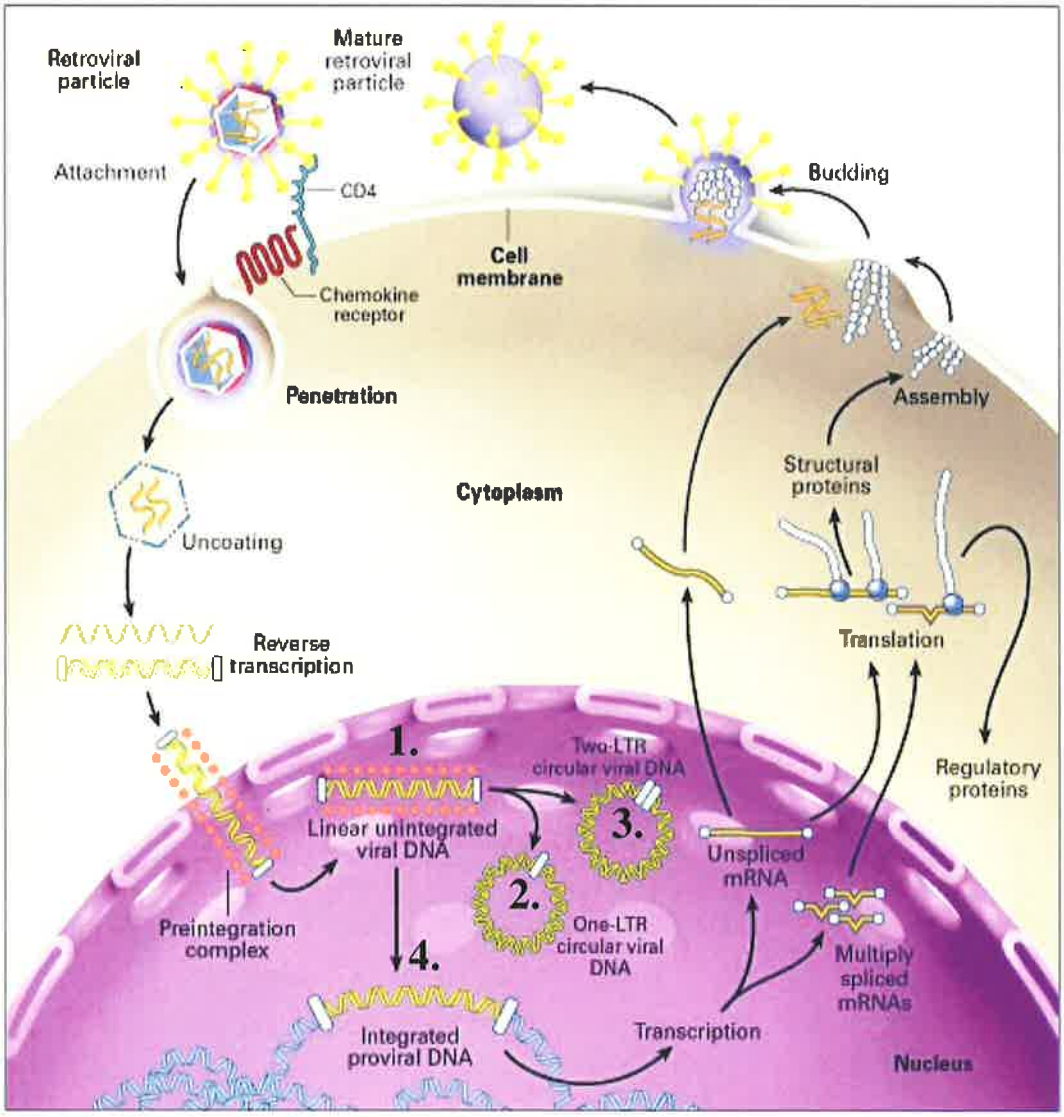
1.2.4.1 Viral attachment and viral fusion

Human CD4+ lymphocytes, macrophages, microglial, dendritic and Langerhans cells, are the natural targets for HIV-1 infection. Generally HIV begins its infection of a susceptible host cell by binding to the CD4+ receptor on the host cells, and then to a co-receptor (Alkhatib et al., 1996, Choe et al., 1996, Wu et al., 1996). CD4 binding follows less specific, adhesion factor-mediated interactions with the cell surface that increase the localized concentration of virions (Mondor et al., 1998). Binding of the HIVgp120 envelope glycoprotein to CD4 induces conformational changes in gp120 that create or expose a binding site for a co-receptor (Trkola et al., 1996, Wu et al., 1996). HIV-1 co-receptors are transmembrane G-coupled proteins belonging to the β-chemokine receptor family. They act together with the CD4 molecule to allow HIV-1 entry into the cell. CXCR4 and CCR5 are two typical representatives of this chemokine receptor family, which have been implicated in HIV-1 infection. Once available, the co-receptor binding site interacts with a complex, discontinuous region of the co-receptor that involves, but is not limited to the amino-terminal domain (Doranz et al., 1997, Dragic et al., 1998, Rucker et al., 1996). The association of gp120 with CCR5 or CXCR4 then drives additional conformational changes within the entire trimeric gp120/gp41 complex leading to the insertion of the gp41 fusion peptide into the host cell membrane, provoking fusion and entry (Kwong et al., 2000). Uncovering the progress how HIV fuses with cell has opened up the possibilities for antiviral drugs development (Deen et al., 1988). Although the predominant cell types infected *in vivo* are T lymphocytes and macrophages which require a high affinity interaction between the trimeric gp120

Fig 1.5 Essential Steps in the HIV-1 Life Cycle

HIV-1 attachment to cells is mediated by viral glycoprotein gp120 and cellular CD4 surface protein receptor. The viral RNA genome then enters the cytoplasm as part of a nucleoprotein complex and is reverse-transcribed into double stranded DNA. Linear double stranded viral DNA is targeted to the cell nucleus as part of a preintegration complex. In the nucleus, unintegrated viral DNA is found in both linear and circular forms. Viral DNA is integrated into the host cell chromosome and serves as a template for viral transcription. Transcription of the proviral DNA template and alternative mRNA splicing create spliced viral mRNA species encoding the regulatory proteins Tat, Rev, and Nef and accessory proteins, and the unspliced viral mRNA encoding the viral structural proteins. All the proteins finally assemble together with genomic viral RNA into virus particles leading to budding and release of infectious retroviral particles from the cell.

Adapted from Furtado et al.(1999)


Retroviral particle
Mature retroviral particle
Attachment
CD4
Chemokine receptor
Cell membrane
Budding
Penetration
Assembly
Structural proteins
Cytoplasm
Uncoating
Reverse transcription
Translation
Regulatory proteins
1.
Two-LTR circular viral DNA
Linear unintegrated viral DNA
3.
2.
Unspliced mRNA
Preintegration complex
One-LTR circular viral DNA
Multiply spliced mRNAs
4.
Integrated proviral DNA
Transcription
Nucleus

protein complex embedded within the virion envelope and the CD4 receptor present on the target cell surface, various other cell types including CD8+/CD4- T lymphocytes, microglial, dendritic and langerhans cells have also been shown to contain HIV-1 and support virus replication to varying degrees (Kawamura et al., 2001, Peterson et al., 2004, Saha et al., 2001) . However, the specific receptors mediating virus entry in these cell types are largely uncharacterised and may include C-type lectins (de Parseval et al., 2004) or phospholipids expressed by host cells or cell surface proteins present on the envelope membrane of primary HIV-1 isolates acquired as viruses exit infected cells (Callahan et al., 2003).

1.2.4.2 Reverse transcription

Following the fusion between the virus and cell membrane, the viral nucleocapsid is released into the cytoplasm. The matrix rapidly dissociates releasing the viral core into the cytoplasm, which then also disintegrates by disassembly of CA core proteins exposing a viral nucleoprotein complex. What triggers these sequential steps leading to a productive replication pathway is not clear. Within an exposed nucleoprotein complex genomic RNA is reverse transcribed into dsDNA by the viral encoded reverse transcriptase, RT (see **1.2.3**). The accepted model for reverse transcription involves: **1**. Binding of host-derived $tRNA^{lys}$ to the viral RNA Primer Binding Site (PBS), after which transcription extends from the $tRNA^{lys}$ to produce minus-strand strong-stop DNA containing R-U5-PBS regions. **2**. The RNase H domain of RT degrades the template RNA molecule as the DNA copy is synthesised. **3**. Strand transfer of single-stranded minus strong–stop DNA to the complementary R sequence located at 3' end of the viral genome. **4.** Minus-strand cDNA synthesis, accompanied by RNase H degradation of the entire RNA genomic template except for PPTs RNA sequences. **5**. The PPT RNA sequences serve as primers to initiate

plus-strand DNA synthesis. **6**. Finally, plus-strand strong-stop DNA encoding the U3- R-U5-PBS region is displaced and re-anneals to the complementary 5' R-U5-PBS sequence permitting extension of both minus and plus strands to generate the double strand DNA including two duplicated U3-R-U5 (Long Terminal Repeat, LTR) regions (Figure1.6).

As described above, reverse transcription of HIV involves two processes, initiation and subsequent elongation reaction. Recent studies show that these processes are highly regulated. This might lead to a difference at the rate of initiation and elongation reaction. Both processes are carried out by a reverse transcription complex composed of (at least) heterodimeric RT, cellular $tRNA^{(lys3)}$ and HIV-1 genomic RNA. A number of viral factors, including Tat (Apolloni et al., 2003), Nef, Vif, Vpr, IN and NCp7 (Harrich & Hooker, 2002), the RNA stem-loop structure TAR, and cellular proteins participate in the RTN complex to play various roles in the initiation and elongation of the reverse transcription reaction. RT lacks proofreading 3'-5'exonuclease activity and therefore reverse transcription results in a high error rate estimated at 1 in 10^4 nucleotide incorporated. This facilitates the emergence of drug resistant HIV-1 strains during anti-viral treatment. RT is a major target for current drug therapy of HIV infection (Mitsuya & Broder, 1987), and understanding the mechanism of reverse transcription will likely lead additional novel antiretroviral approaches.

1.2.4.3 Viral DNA integration

Retroviral integration, in which newly reverse-transcribed viral DNA is inserted into the host cell chromosome, is essential for productive infection (Englund et al., 1995, Sakai et al., 1993a, Stevenson et al., 1990). Once the genetic material of HIV has

Figure 1.6 A reverse transcription model for HIV

A. The primer for initiation of (-) strand viral DNA synthesis is a cellular tRNA bound to the 5' terminal primer-binding site (PBS) of virion RNA. Reverse transcriptase (RT) elongates the (-) strand synthesising minus strand strong-stop DNA, while degrading the RNA template by an RNase H activity.

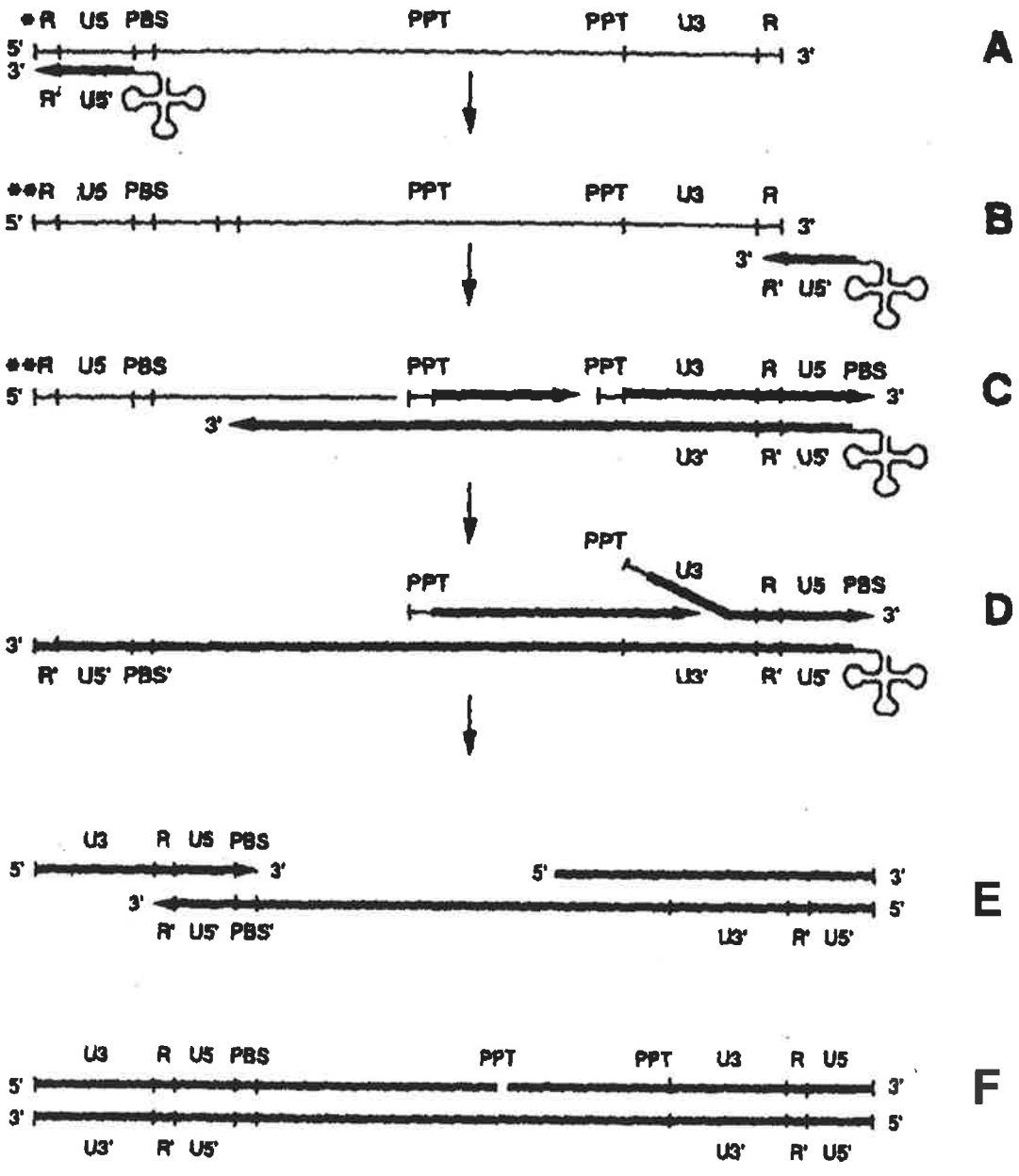
B. Strand transfer of ssDNA to the 3' termini of the viral genome, forming a duplex with the complementary R sequence.

C-D. RT continues to elongate the (-) strand and degrades (+) strand RNA by RNase H activity except for stable polypurine tract (PPT) sequences in the viral RNA. PPT duplexed RNA initiates (+) strand DNA synthesis by RT. (-) strand DNA is completed

E. (+) Strand strong-stop DNA displaced from the 3' PPT transfers and hybridizes to the 5' complementary RU5 PBS sequence.

F. Both complementary DNA strands are completed by RT

Modified from Li et al.(1993)


*R U5 PBS
PPT
PPT U3 R
5'
3'
3'
R' U5'
A
**R U5 PBS
PPT
PPT U3 R
5'
3'
3'
R' U5'
B
**R U5 PBS
PPT
PPT U3 R U5 PBS
5'
3'
3'
U3' R' U5'
C
PPT
PPT
U3
R U5 PBS
3'
3'
R' U5' PBS'
U3' R' U5'
D
U3 R U5 PBS
5'
3'
5'
3'
3'
5'
R' U5' PBS'
U3' R' U5'
E
U3 R U5 PBS
PPT
PPT U3 R U5
5'
3'
3'
5'
U3' R' U5'
U3' R' U5'
F

been reverse transcribed into DNA, the HIV preintegration complex composed of both viral and cellular proteins closely associated with viral DNA is transported into the cell nucleus where it can be integrated into cellular chromosome. To achieve this, the double stranded linear HIV DNA molecule undergoes specific processing: **1**) Removal of a dinucleotide from the 3' termini of the linear viral DNA molecule. **2**) Covalent linkage of the 3' viral DNA termini with precleaved host cellular DNA and **3**) Trimming of 5'- ends of the viral DNA and ligation with the host cell DNA. This process is controlled by the action of the HIV viral integrase. Once the viral DNA is integrated into the host cell chromosome, the HIV genetic material is implanted permanently within the cell, but may or may not be expressed or HIV may persist in a latent state for many years. This ability of HIV to persist latently in cells is a major barrier to complete eradication of HIV in patients. Considering the essential role IN has in establishing a HIV infection, HIV integrase has been a more recent target for HIV drug treatment (Farnet & Bushman, 1996, Hong et al., 1997). This will be discussed in the latter part of this chapter.

1.2.4.4 Transcription and translation

HIV-1 requires transcription of its provirus genome for completion of the replication cycle and production of progeny virions. Transcription from the HIV genome requires the 5' long terminal repeat which contains the viral promoter, and involvement of the virus encoded regulatory proteins, Tat and Rev. The process is also dependent on cellular proteins and therefore regulated by the metabolic state of the cell. Details describing transcription are presented in section **1.2.5.2.**

Synthesised mRNA is spliced and transported to the cell cytoplasm for protein synthesis in a highly regulated manner. Multiple splicing patterns result in the

production of more than 30 distinct viral RNA species, classified in three main groups: **1**) fully spliced; **2**) singly spliced and **3**) unspliced. Completed spliced transcripts predominate early in infection and encode the viral regulatory proteins Tat, Rev and Nef. Singly spliced and unspliced viral RNAs encoding viral structural proteins and new viral genomes respectively appear "late" in the viral replication cycle. A detailed review of RNA translation is described in **1.2.6**

1.2.4.5 Assembly of virus and viral budding

HIV particles assemble at the cell plasma membrane and are released by budding from the cell surface. Assembly is controlled primarily by the Gag protein, which recruits all the building blocks required for the formation of a fully infectious virion. In the case of HIV this includes both viral and cellular components. Assembly is dependent on the action of (1) an aspartyl protease encoded by the viral *pol* gene, which is responsible for cleavage of the gag and gag-pol precursors into mature proteins (Kohl et al., 1988, Peng et al., 1989) and (2) cellular N-protein myristoyl transferase (NMT) which adds myristic acid to the N-terminus of Gag, Gag-pol and Nef viral polyprotein precursors (Gottlinger et al., 1989). By virtue of the N-terminal myristoylation reaction, polyproteins are imported into the endoplasmic reticulum (ER) and targeted to the cell membrane (Hermida-Matsumoto & Resh, 2000, Shiraishi et al., 2001). The assembly of these structural polyproteins at the cell surface and association with two strands of unspliced viral RNA allows formation of a core precursor structure. Gag is considered to provide the principal driving force for virus assembly, as illustrated by the fact that HIV Gag can efficiently form virus-like particles even when expressed in the absence of other viral proteins (Gheysen et

al., 1989, Harvey et al., 2003). A Gag cleavage product p6 plays an important role in virion budding and release (for details refer to section **1.2.6**).

1.2.5 HIV gene expression

The HIV genome contains 9 genes, three of which, *tat, rev* and *nef*, are classified as "early" and are expressed independently of the Rev action; and six of which are classified as "late" genes (Pandori et al., 1996, Schwartz et al., 1995a) *gag, pol, env, vpr, vpu* and *vif*, and require Rev for their expression in the cytoplasm. Regulation of viral gene expression is accomplished by the concerted action of cellular and viral protein functions.

1.2.5.1 HIV proviral genome transcription - overview

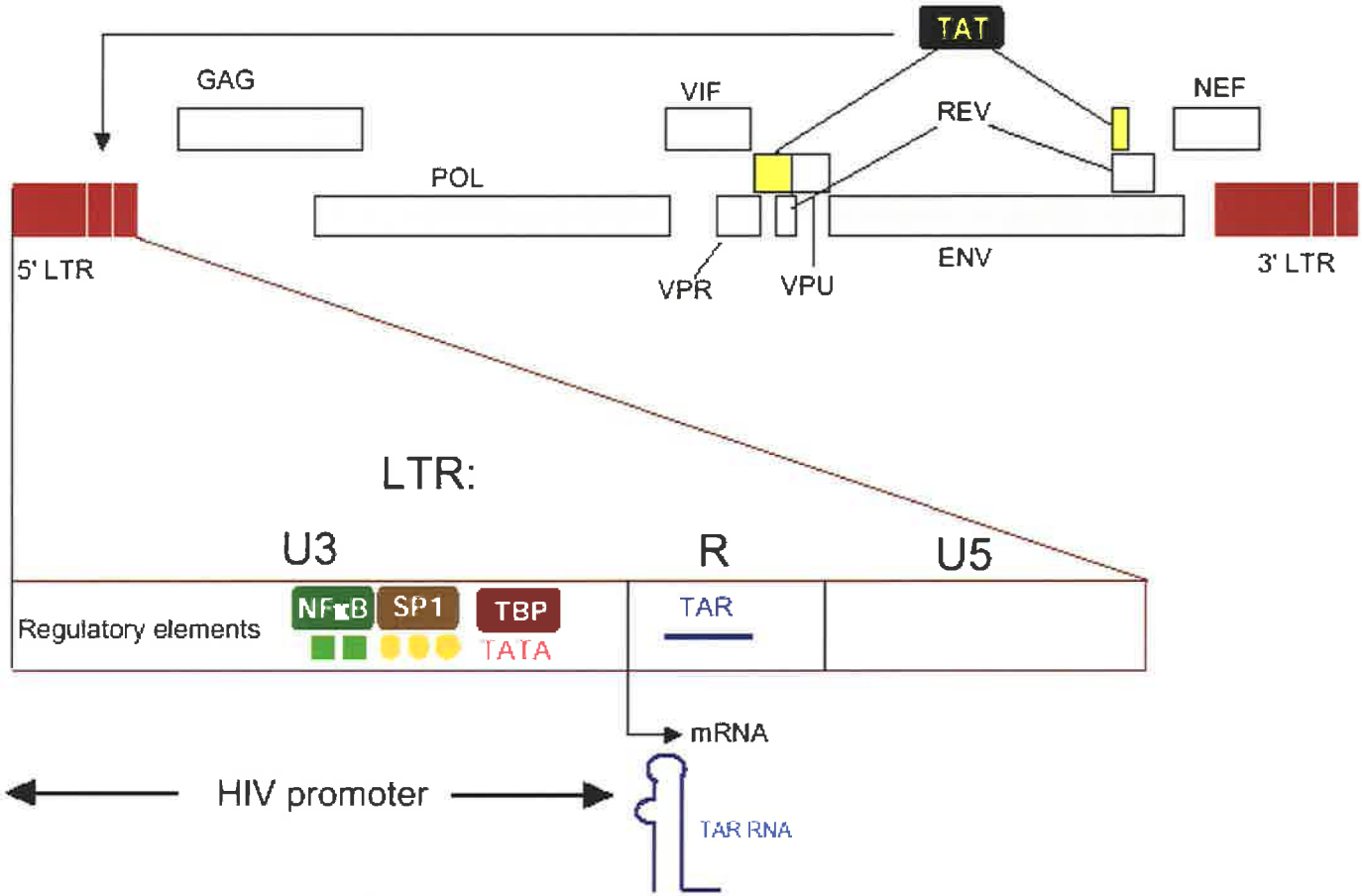
As described in section 1.2.3, HIV RNA is around 9.2 kilobases in length, while the integrated form of HIV DNA, also known as the provirus, is approximately 9.8 kilobases in length. At each end of the provirus is a repeated sequence known as the Long Terminal Repeat or LTR (Figure1.7). Viral transcription is initiated from a single promoter located in the 5'LTR and is regulated by a number of cis-acting elements that interact with transcription factors including NF-*k*B SP-1 and NF-AT to regulate the recruitment of RNA polymerase II necessary for transcription initiation. The full-length primary HIV-1 transcript contains multiple splice donor (5'splice site) and splice acceptor (3'splice site) sites, which can be processed to yield more than 30 alternative mRNAs divided into 3 groups (Purcell & Martin, 1993, Schwartz et al., 1990):

1) Multiply spliced, which are 2kb in length expressing Rev, Nef, and the two-exon form of Tat. These mRNAs exit from the nucleus independently of Rev.

Figure 1.7 Regulatory elements in the HIV-1 LTR

The HIV-1 LTR is divided into three functionally distinct regions designated U3 (-453 to +1), R (+1 to +98) and U5 (+99 to +185). U3 region functions as viral promoter containing several regulatory elements represented by squares or circles. The R region encodes the RNA sequence, which forms the Tat activation region, TAR.

Adapted from the website http://misa.sus.mcgill.ca/presentations/Gatignol1.pdf


TAT
GAG
VIF
REV
NEF
POL
5' LTR
VPR
VPU
ENV
3' LTR
LTR:
U3
R
U5
Regulatory elements
NFκB
SP1
TBP
TATA
TAR
mRNA
HIV promoter
TAR RNA

2) Unspliced, which are translated into Gag and Gag-Pol precursor proteins, or serve as genomic RNA for packaging into virions. The unspliced mRNA, 9 kb in length, is dependent on Rev for export from the nucleus to the cytoplasm.
3) Partially spliced, which are heterogeneous 4-5kb in length and retain the second intron of HIV. They have the potential to express Env, Vif, Vpu and Vpr and also require Rev for export (Figure1.8).

1.2.5.2 Regulation of transcription

Infection by HIV-1 often results in a period of latency characterised by variable low-level virus production from the integrated provirus. The integrated provirus is subject to regulation by viral regulatory proteins and cellular transcription factors. In addition, a variety of environmental stimuli such as ultra violet light, heat shock, and oxygen radicals may activate proviral transcription (Duh et al., 1989, Kalichman, 1998, Nabel & Baltimore, 1987, Tong-Starksen et al., 1987, Valerie et al., 1988). The exact mechanism by which these stimuli activate HIV gene expression is not fully understood, but evidence suggests the activation of the HIV-1 LTR is mediated by cytokines and environmental stress and requires an active CSBP/p38 MAP kinase (Kumar et al., 1996).

After integration in the host cell genome, the HIV-1 provirus is packaged into chromatin. Modification of chromatin within the vicinity of an HIV-1 provirus may also be involved in virus gene regulation. Modifications, including a specific chromatin disruption occurring in the HIV-1 promoter during transcriptional activation, can alter the accessibility of DNA to specific transcription activators or repressors, general transcription factors and RNA polymerase (Van Lint et al., 1996).

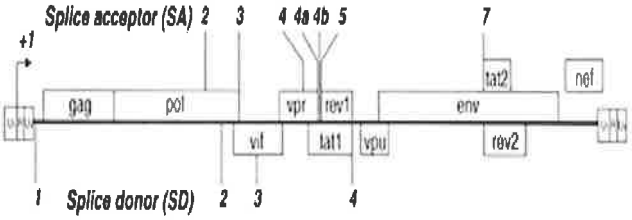
Fig 1.8 Genomic organization and mRNA production by HIV-1

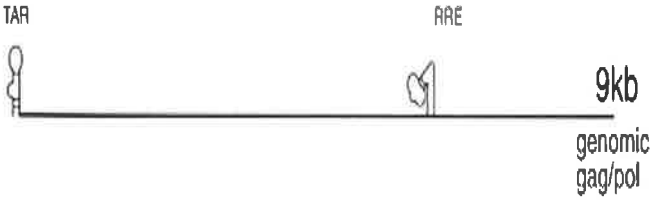
Upper panel: Schematic representation of the 9.7kb proviral genome with its long terminal repeat sequences (LTR) specifying the locality of genes, splice acceptor (SA) sites (above), splice donor (SD) sites (below) and start of transcription ($^{+}1$).

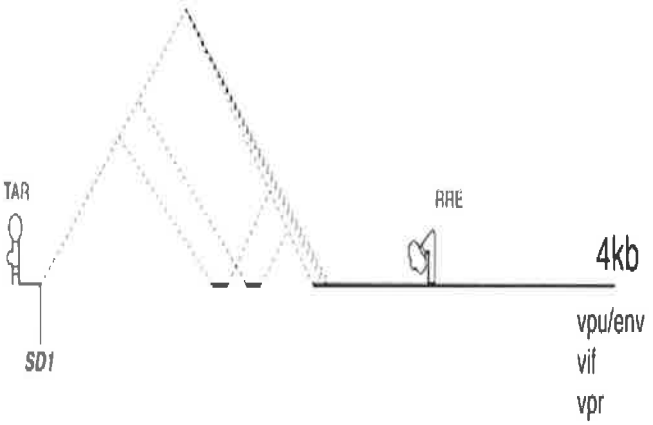
Lower panels: The three classes of viral RNA produced during HIV-1 infection include:

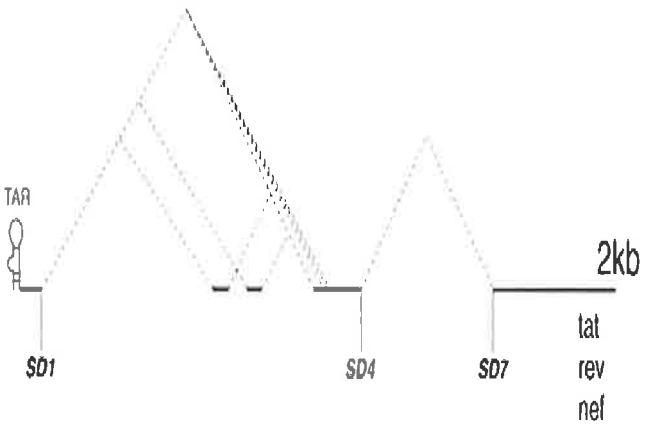
(i) unspliced 9kb transcript that forms either new viral genomes or mRNA encoding Gag and Gag/Pol polyproteins , (ii) singly spliced 4kb mRNA encoding Vpu/Env polyprotein , Vif and Vpr and (iii) multiply spliced 2kb mRNA encoding Tat, Rev and Nef. Dashed lines represent intron sequences, which are spliced. Variations in splicing in the 5' region of 2kb and 4kb mRNA occur because of the alternative use of several SA sites. Only the basic structure of many different 4kb and 2kb mRNAs is shown.

Adapted from Davis, Adam (Ph.D thesis)


Splice acceptor (SA) 2 3 4 4a 4b 5 7
+1
gag
pol
vpr
rev1
env
tat2
nef
vif
tat1
vpu
rev2
1
Splice donor (SD) 2 3 4


TAR
RRE
9kb
genomic
gag/pol


TAR
RRE
4kb
SD1
vpu/env
vif
vpr


TAR
2kb
SD1
SD4
SD7
tat
rev
nef

Emerging data now suggest that the dynamic regulation of chromatin structure in the vicinity of the LTR promoter adds an additional level of complexity to the regulation of HIV expression (He et al., 2002).

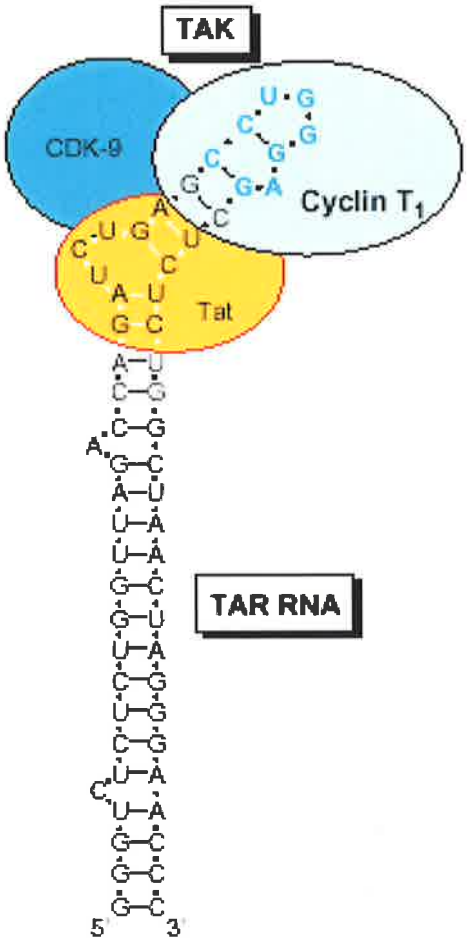
The viral protein Tat is essential for regulation of HIV-1 proviral transcription. Kao *et al* demonstrated that short, truncated transcripts predominate in the absence of Tat, and that in the presence of Tat there is a dramatic increase in the levels of long transcripts (Kao et al., 1987, Kessler & Mathews, 1992, Ratnasabapathy et al., 1990, Toohey & Jones, 1989) and an increasing rate of transcriptional initiation (Laspia et al., 1989a). Tat interacts with a Tat activation region (TAR) at the 5' termini of all primary viral transcripts. Binding of Tat to a U-rich bulge near the apex of the TAR RNA stem and the G26:C39 base pair immediately above the bulge, results in the association with Tat of a protein kinase complex, TAK (Tat-associated kinase) (Herrmann et al., 1996, Herrmann & Rice, 1995). TAK carries a kinase subunit CDK9, and a cyclin subunit called cyclin T1 (Wei et al., 1998) which regulates CDK9 activity and enhances the association of Tat with TAR RNA (Figure1.9). A functional TAR RNA/Tat/CyclinT1/CDK9 is necessary for phosphorylation of the RNA Polymerase II carboxy terminal domain (CTD). Phosphorylation of RNAPII CTD promotes transcriptional initiation, elongation and rapid reinitiation (Kobor & Greenblatt, 2002).

The viral protein Rev exports unspliced and partially spliced HIV RNA into the cytoplasm for translation. Before a "threshold" level of Rev required for this function is reached, viral transcripts are multiply spliced by the cellular machinery and exported from the nucleus normally. Nuclear export of unspliced and partially

Figure 1.9 Recognition of TAR RNA by Tat and TAK.

Tat recognition primarily requires interactions with the bulge region of TAR. In the presence of cyclin T1, conformational rearrangements in Tat permit interactions with the apical loop sequences. Part of the interface between Tat and cyclin T1 is believed to involve cysteine residues from each protein that participate in zinc binding

Adapted from Karn (1999)


TAK
CDK-9
Cyclin T1
Tat
TAR RNA
5'
3'

spliced mRNAs depends upon the binding of multiple copies of Rev at a cis-acting, highly structured RNA target or rev response element (RRE) in the envelope region of the transcript (Malim & Cullen, 1991, Sandri-Goldin, 2004). Rev contains an N-terminal arginine-rich sequence that serves as both a nuclear localization signal (NLS) and RRE-specific RNA binding domain, and this motif is flanked by sequences that mediate Rev multimerization at the RRE (Malim & Cullen, 1991). A leucine-rich motif present in the carboxy terminal of Rev serves as a nuclear export signal (NES) (Figure1.10) (Fischer et al., 1995a). This NES is essential for Rev binding to CRM1, a nuclear export factor belonging to the importin/exportin or karyopherin family of nuclear transport receptors (Fornerod et al., 1997, Sandri-Goldin, 2004, Stade et al., 1997). The 3D structure of rev is not known yet. However, the hydrophilic NLS is likely to be more exposed than the hydrophobic NES. Therefore, when rev is synthesized in the cytoplasm, the importin α/β may bind to its NLS, carrying it into the nucleus. When rev is in the nucleus, it can bind to mRNA's RRE. The RNA binding domain of rev is in the same region as NLS. Thus, upon binding to mRNA, the NLS of rev is masked, exposing only NES. Cooperation between GTPase Ran, which is a small GTPase that is required for protein import, mRNA export, and the maintenance of nuclear structures, and Rev-CRM1 results in the translocation of viral mRNAs through the nuclear pore complex (Sandri-Goldin, 2004).

1.2.6 HIV encoded proteins and their functions

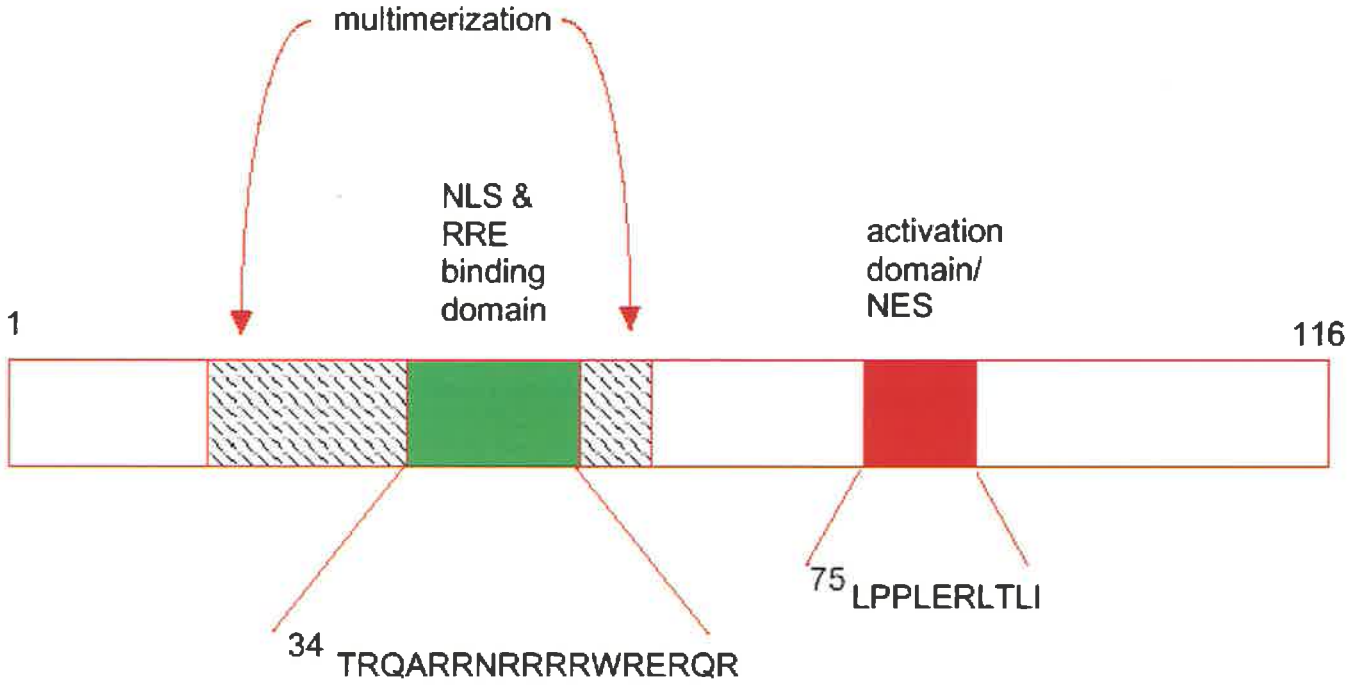
1.2.6.1 The major structural proteins:

Gag: *gag* encodes a 55-kilodalton (kDa) precursor polyprotein $p55^{Gag}$ translated from unspliced viral mRNA. Gag contains all the properties necessary for membrane

Figure 1.10 Functional domains of Rev

Three essential functional domains of Rev are indicated by shaded region. Amino acid sequences of the N-terminal domain contains a basic Arginine-rich sequence essential for Rev binding to the Rev response element (RRE) and a hexapeptide sequence that functions as a nuclear localization signal (NLS). Amino acids flanking the basic domain are required for multimerization of Rev monomers. Within the C-terminal domain a Leucine-rich sequence functions as a nuclear export signal (NES)

Adapted from Pollard & Malim (1998)


multimerization
NLS &
RRE
binding
domain
activation
domain/
NES
1
116
34 TRQARRNRRRRWRERQR
75 LPPLERLTLI

REV

targeting, viral particle assembly, and budding from the host cell (Wills & Craven, 1991). During translation, the N terminus of Gag is myristoylated (Bryant & Ratner, 1990), which is essential for targeting it to the cell cytoplasmic membrane. Membrane associated Gag recruits two copies of viral genomic RNA and other viral and cellular proteins that trigger budding of the viral particle from the surface of the infected cell. After budding, subsequent morphogenesis of the mature virus involves the proteolytic processing of the Gag polyprotein into MA (matrix p17), CA (capsid p24), NC (nucleocapsid p7) and p6 (Gottlinger et al., 1989). This proteolytic cleavage, carried out by PR, autoproteolytcally cleaved from Pol polyprotein, facilitates maturation of the virus into infectious virions (Gelderblom et al., 1987). MA proteins associate to form a shell encapsidating the viral core of the mature virus. However 1% of MA proteins are phosphorylated by an intravirion kinase resulting in incorporation of MA into the viral nucleoprotein complex contained within the conical core. Upon virus entry, the MA shell dissociates while MA in the nucleoprotein complex participates in nuclear targeting of the complex via a nuclear localisation sequence (NLS) in MA (Gallay et al., 1995). The p24 protein forms the conical core of the viral particles. An interaction between Cyclophilin A and the p24 region of p55 has been demonstrated to be essential for viral replication (Franke & Luban, 1996). NC binds to the viral RNA packaging signal through interactions mediated by two zinc-finger motifs and facilitates viral RNA packaging (Lapadat-Tapolsky et al., 1993). The p6 polypeptide region is thought to mediate the incorporation of the accessory protein Vpr, into assembling virions (Paxton et al., 1993). As mentioned in section **1.2.4.5**, p6 also facilitates the efficient release of budding virions from the cell surface and/or from each other. Studies of mutant defective in p6 demonstrated assembled particles remaining attached to the

extracellular surface of the plasma membrane via a thin membranous stalk (Gottlinger et al., 1991). This release defect was seen in adherent cell lines and in monocyte-derived macrophages (Demirov et al., 2002, Gottlinger et al., 1991). In contrast, in T cell lines and in primary lymphocytes the major defect of p6 mutants appears to be at the level of virion-virion detachment (Demirov et al., 2002). A defect in virus separation may also account for the observation that HIV-1 particles lacking p6 appear very large as assayed by sucrose gradient sedimentation (Garnier et al., 1999, Garnier et al., 1998).

Gag-Pol and the viral enzymes: The Gag-Pol precursor polyprotein, p160 is generated by a ribosomal frame shifting event during translation of Gag, which is triggered by a specific cis-acting RNA motif (Parkin et al., 1992). When ribosomes encounter this motif they shift with a frequency of 5% to the *pol* reading frame without interrupting translation. Consequently the ratio of Gag and Gag-Pol polyproteins produced in infected cells is approximately 20:1. $Pr160^{gag\text{-}pol}$ is incorporated into viral particles by interacting with the N-terminal domain of assembling $Pr55^{gag}$ (Huang & Martin, 1997, Srinivasakumar et al., 1995). $Pr160^{gag\text{-}pol}$ is proteolytically cleaved into protease (PR, p10), integrase (IN, p31), and reverse transcriptase (RT, p66/51), initially by autoproteolytic cleavage of PR followed by viral PR mediated cleavage of the remaining protein. RT functions as a RNA- and DNA- dependent DNA polymerase during reverse transcription. RT is an active heterodimer of two polypeptides: RT p66 and RT p51, in which only the p66 polypeptide contains the polymerase activity centre and encodes RNase H activity necessary for degradation of the RNA template during first DNA strand synthesis. IN mediates the integration of HIV DNA into the genomic DNA of an infected cell.

Env: *Env* encodes a 160 kDa polyprotein, expressed from a singly spliced mRNA transcript. A cellular protease cleaves gp160 to generate gp41 and gp120. gp120 expressed on the surface of the virion interacts with the cellular receptor, CD4 (Landau et al., 1988). gp41 is a transmembrane protein that binds to gp120 non-covalently (Bernstein et al., 1995). gp120 has nine highly conserved intrachain disulfide bonds and five hypervariable regions, designated V1 to V5, whose amino acid sequence can vary greatly among HIV isolates (Kwong et al., 1998). One such region, called the V3 loop, is not involved in CD4 binding, but is an important determinant of HIV tropism for T lymphoid cell lines and primary T cells and macrophages (Hwang et al., 1991). This specificity is the result of an interaction between the V3 loop and HIV co-receptors CXCR4 or CCR5, which belong to the family of β-chemokine receptors present on susceptible cells (Deng et al., 1996, Feng et al., 1996). For this reason, the V3 loop is also the principal target for neutralizing antibodies that block HIV-1 infectivity (Goudsmit et al., 1988).

1.2.6.2 Regulatory proteins/accessory proteins:

In addition to structural proteins the HIV genome encodes six regulatory and accessory proteins. The regulatory proteins Tat and Rev are absolutely necessary for virus replication. In contrast, Nef, Vpu, Vif, Vpr and the HIV-2 and SIV Vpx proteins are dispensable for virus replication *in vitro* and hence termed accessary proteins. Nevertheless, the high degree of conservation of these proteins reveal that they fulfil crucial functions *in vivo* (Trono, 1995). Investigation of the molecular mechanisms employed by HIV accessory proteins may yield new approaches for therapeutic strategies.

Tat: Tat is a small nuclear protein of 86 to 101 amino acids, depending on the viral strain, encoded by two separate exons (see Figure1.4). Analyses of "full-length" Tat have been performed commonly using the 86 amino acid version. However, it should be noted that while a few laboratory strains (e.g. HXB2 and NL4.3) have the truncated Tat (86aa), most HIV-1s have the 101 aa protein. The domain structure of Tat is typical of a transcriptional activator including an activation domain and a nucleic acid binding domain. Tat function is dependent on its interaction with a bulged RNA stem-loop structure, TAR (Tat activation region) that is present at the 5-terminus of all viral mRNAs. Despite intensive efforts, the mechanism of Tat action remains incompletely understood. As discussed before, Tat is a major transcriptional activator of the proviral LTR promoter element and is essential for viral replication in almost all culture systems (Cullen, 1998, Laspia et al., 1989b, Rice & Mathews, 1988). One mechanism used by Tat to activate the HIV-1 LTR involves increasing the processivity of RNA polymerase II molecules and hence the efficiency of transcriptional elongation (Cullen, 1998). Other studies have also demonstrated that HIV-1 Tat protein specifically interacts with and activates cyclin-dependent kinases (Cujec et al., 1997, Garcia-Martinez et al., 1997, Wei et al., 1998) which phosphorylate the C-terminal domain of RNA polymerase II and increase HIV-1 gene expression. Ulich *et al* demonstrated that the amino terminus of Tat is critical for its role in modulating HIV-1 reverse transcription (Ulich et al., 1999).

More recently HIV-1 protease PR regulation of Tat activity has been shown to be essential for efficient reverse transcription and replication. Using a rabbit reticulocyte lysate (RRL) system, Apolloni showed that Tat was specifically cleaved in the presence of HIV protease PR, producing a protein of approximately 5 kDa. This suggested that the cleavage site was located in or near the Tat basic domain (amino

acids 49 to 57), which the same group has previously shown to be important in reverse transcription. This PR cleavage was also observed in cell culture. Interestingly, the extent of Tat cleavage by PR correlated with its ability to support efficient reverse transcription implying that the PR cleavage is important for HIV efficient reverse transcription (Apolloni et al., 2003).

Rev: Rev is encoded by two exons (Figure1.4) and accumulates within the nuclei of infected cells (Kim et al., 1989). Rev binds to a 240-base region of complex RNA secondary structure, termed the Rev Response Element (RRE), that is present in all unspliced and partially spliced viral transcripts. Normally, RNAs that contain introns (i.e., unspliced or incompletely spliced RNA) are retained in the nucleus. Binding of Rev to RRE facilitates export of unspliced and partially spliced viral RNAs from the nucleus to the cytoplasm. In addition to the RRE binding domain, Rev also contains at least two other functional domains, a multimerisation domain and an effector domain. Rev is believed to exist as a homo-tetramer in solution. The effector domain is a specific nuclear export signal (NES) located between residues 75 and 84 (see Figure1.10) (Fischer et al., 1995b). Using the yeast two-hybrid assay, nucleoporins have been demonstrated to bind Rev thereby suggesting their involvement in the biological activity of the Rev NES (Cullen, 1998). High levels of Rev expression can lead to the export of so much intron containing viral RNA that the amount of RNA available for complete splicing is decreased, which, in turn, reduces the level of Rev expression. Therefore, this ability of Rev to decrease production of fully spliced viral RNA ultimately reduces the amount of Rev protein (Felber et al., 1990). Rev is absolutely required for HIV-1 replication: proviruses that lack Rev function are

transcriptionally active but do not express viral late genes and thus do not produce virions.

Vpu: Vpu is a small transmembrane protein that has at least two independent activities. It induces the degradation of newly translated CD4 molecules (Paul & Jabbar, 1997), and increases the release of virus particles from infected cells (Terwilliger et al., 1989). While CD4 degradation is mediated by sequences located in the Vpu cytoplasmic tail, enhanced virion release is dependent on the hydrophobic amino-terminal transmembrane domain. Vpu acts to facilitate virion release by promoting the budding of virions from cellular plasma membrane, as opposed to intracytoplasmic membrane structures (Bour & Strebel, 2003, Klimkait et al., 1990)

Vpr: Vpr, another HIV accessory protein is a late HIV gene product, 96 amino acids in length, packaged into the virion nucleocapsid in equimolar amounts to that of Gag protein. As described in section **1.2.6.1**, Gag^{p6} protein mediates the incorporation of Vpr into assembling nucleocapsids. During the early stage of HIV-1 replication Vpr forms part of the preintegration complex (PIC). Sequence analysis has identified Vpr as a nucleophilic component that influences nuclear localisation of viral nucleic acids in nondividing cells (Heinzinger et al., 1994). Vpr hence may play a role in mediating the nuclear import of HIV-1 PICs into the nucleus of non-dividing cells. Another interesting activity of Vpr is its ability to inhibit cell division of all cells assayed so far including. *E.coli*, yeast, mammalian cells and even human cancer cells. Several different models were proposed to explain how Vpr has so many effects on the cell division cycle. Vpr might disrupt multiple targets, each of which controls a different process or pathway. Alternatively, Vpr may target a single

protein or organelle that affects these interdependent processes (Chang et al., 2004, Zhang et al., 1997).

Nef: Nef is a 27-kDa myristoylated protein that is encoded by a single exon that extends into the 3' LTR (see Figure1.4). Nef is the first viral protein to accumulate to detectable levels in a cell following HIV-1 infection (Kim et al., 1989). Nef is the largest accessory protein of 206 amino acids in length, and expressed at greater levels than Tat and Rev. Nef was initially found to exert a negative effect on the rate of HIV-1 replication in culture. Nef has been shown to play at least three important functions in HIV-1 infection.

1) Nef downregulates cell surface expression of CD4 and major histocompatibility complex (MHC) class I molecules (Garcia & D., 1992, Garcia & Miller, 1991); Hence as CD4 is the primary receptor for HIV-1, down-regulation of CD4 reduces superinfection of an infected cell and down regulation of class I MHC may help infected cells avoid CTL surveillance. In addition, it has been suggested that virus spread may be inhibited by the inability of progeny virions to effectively dis-engage from infected cells that express high levels of cell-surface CD4 (Marshall et al., 1992). Therefore, downregulation of CD4 expression by Nef may facilitate the release of HIV-1 virions (Benson et al., 1993).
2) Nef perturbs T cell activation also shown in studies of Jurkat T cells where Nef expression inhibited induction of transcription factor NF-*K*B, and prevented antigen receptor-mediated induction of IL-2 expression (Luria et al., 1991). In contrast, results obtained in Nef transgenic mice revealed that Nef led to elevated T cell signalling (Skowronski et al., 1993). The expression of a CD8-Nef chimerical molecule in Jurkat cells had either positive or negative effects on T

cell activation depending on the cellular localization of hybrid Nef molecule (Baur et al., 1994). Consistent with these results, Nef has been found to associate with several different cellular kinases that are present in helper T lymphocytes, such as Tyrosine kinase lck, a serine/threonine kinase.

3) Nef enhances virion infectivity demonstrated by both tissue culture infectious dose analysis, and single-cell HIV infection assay (Miller et al., 1994). Schwartz O *et al* showed HIV-1 reverse transcription in infected cells generated 5- to 10-fold less DNA when the virions were produced in the absence of Nef, indicating that these particles performed reverse transcription in a suboptimal environment (Schwartz et al., 1995b). Other studies also demonstrated that Nef functioned in the process of virion assembly and could not be complemented by expression of Nef in target cells. The enhancement effect of Nef on viral infectivity might be 10-fold or more when primary T cells or certain highly susceptible target cells, such as CD4-Hela cells were infected (Cullen, 1998, Lundquist et al., 2002, Stoddart et al., 2003).

In addition, a lack of Nef function appeared to affect HIV-1 pathogenesis *in vivo*. Individuals infected with a strain of HIV-1 that contained inactivating mutations in Nef, underwent a less progressive disease leading to longer asymptomatic clinical phase of the disease (Deacon et al., 1995, Kirchhoff et al., 1995). This observation provides a significant link between viral genotype and disease progression.

Vif: Vif was one of the least understood HIV viral accessory proteins at the commencement of this doctoral project in early 2001. There has been a remarkable

expansion of body of knowledge regarding the functional aspects of Vif in recent years. Background discussion on HIV Vif is described below.

1.2.7 HIV-1 virion infectivity factor (Vif)

Vif is a late HIV-1 gene product encoded by a singly spliced mRNA expressed in a Rev dependent manner. The mechanism of action of Vif is not fully understood, yet it is clearly important for infectivity (Cullen, 1998) and elucidation of Vif's role in HIV-1 infection may provide an important target for therapeutic intervention.

1.2.7.1 HIV-1 Vif localisation and biological activity

Vif is a highly basic, 23 kDa, 192 amino acid phosphorylated protein synthesised at relatively high levels late in the viral life cycle (Garrett et al., 1991, Schwartz et al., 1991). Immunofluorescence analysis of the subcellular distribution of Vif indicates that it is predominantly localised within the cytoplasmic region, with only weak staining of the nucleus (Goncalves et al., 1995, Karczewski & Strebel, 1996). Indirect immunofluorescence staining assays indicated that Vif has an affinity for microsomal membranes (Goncalves et al., 1994, Goncalves et al., 1995). Simon *et al.* also showed Vif present in the cell cytoplasm and at the cell membrane, and found it to co-localize with the major HIV structural protein, Gag by confocal microscopy (Simon et al., 1999a, Simon et al., 1997).

Vif is highly conserved in HIV-1 and all other lentiviruses with the exception of equine infectious anemia virus (Oberste & Gonda, 1992). Vif is required for HIV-1 replication in primary T-lymphocytes and monocytes/macrophages, while the requirement for Vif differs among different cell lines. This has led to the description

of primary cells and certain cell lines as non-permissive (H9, HuT-78, A3.01) semi-permissive (CEM-ss) or permissive (HeLa CD4, 293T, Cos-7, SupT1), for the replication of Vif-defective viruses (Gabuzda et al., 1992, Sova & Volsky, 1993). Vif acts at a late stage of the virus life cycle during assembly and/or maturation in producer cells, to enhance infectivity of progeny virions 100-1000 fold in non-permissive target cells (Blanc et al., 1993, Fisher et al., 1987, Strebel et al., 1987).

Since Vif does not show any sequence similarity or homology to any other protein it has been difficult to predict its mode of action or the functional domains of Vif. While the N-terminus and C-terminus of Vif appear to be important for binding to viral RNA and Gag respectively, the exact residues mediating this interaction have not been defined (Zhang et al., 2000a). More recently work using in vitro GST fusion proteins, co-immunoprecipitation and the yeast 2-hybrid system has shown that Vif is likely to act as a multimeric protein. The region of Vif mediating this interaction appears to lie in a proline enriched, positively charged fragment at amino acid position 151-164 (Yang et al., 2001).

1.2.7.2 Vif is required for efficient reverse transcription

Analyses of the protein content and morphology of Vif-defective virus particles have been conflicting. Early research on Δ*vif* HIV-1 virions derived from permissive and non-permissive cells revealed little differences in protein composition, RNA content, or reverse transcriptase activity compared to wild type (WT) virions (von Schwedler et al., 1993). In contrast, other studies have indicated that Vif plays a role in increasing Gag protein processing or in incorporation of processed Gag products into mature virions. The presence of unprocessed precursor Gag polyprotein (Pr55gag) and other Gag processing intermediates in PBL-derived Vif-negative extracellular

particles was proposed to contribute to the reduced infectivity of this virus (Sova et al., 2001, Volsky et al., 1995). However, more recent studies did not reproduce these results and no differences in either viral protein composition or particle morphology were detectable (Fouchier et al., 1996, Soll, 1997) pointing to more subtle differences being responsible for the reduced infectivity of *vif*-defective virus particles.

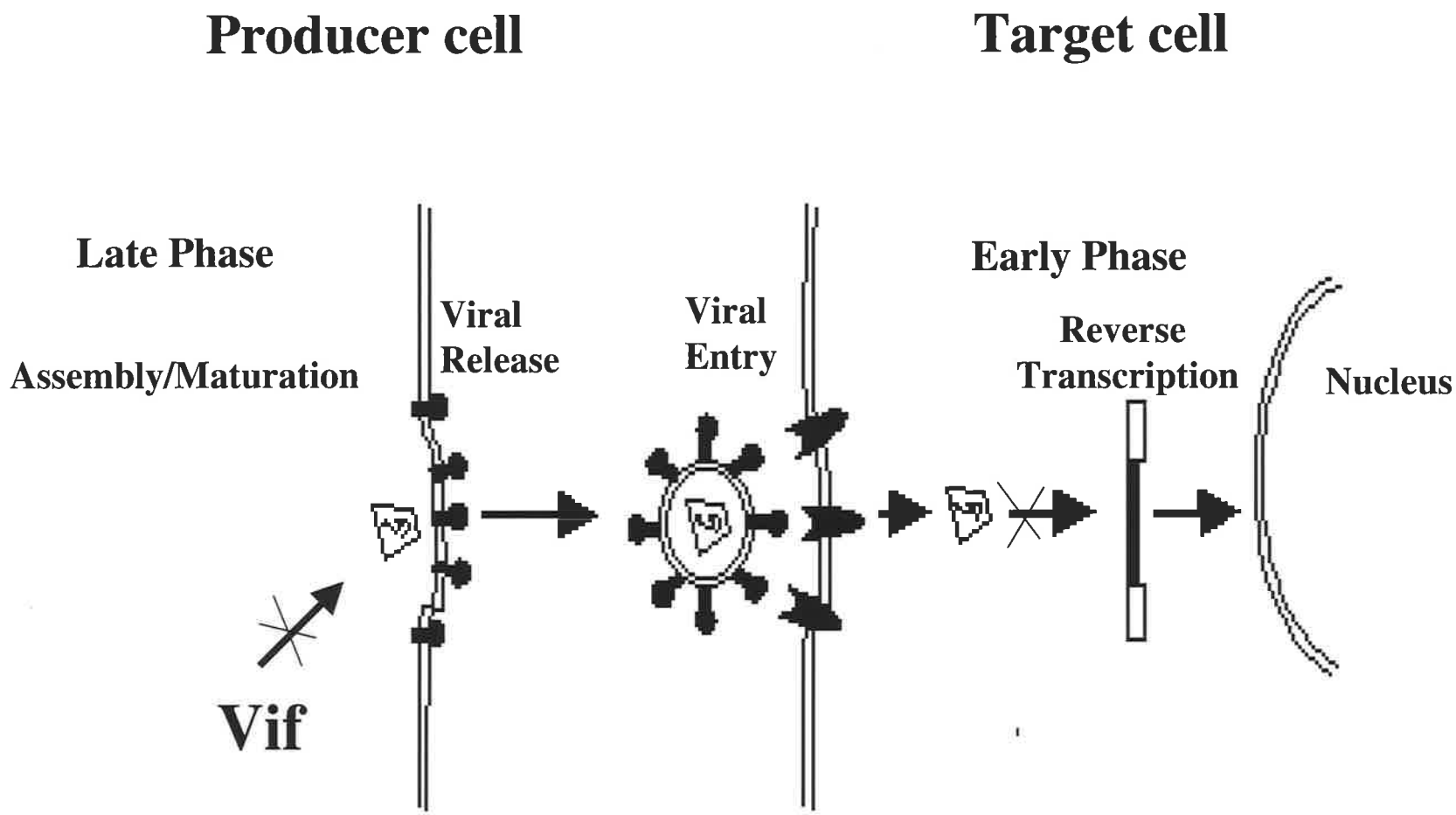
Δ*vif* HIV-1 virus derived from non-permissive cells was reported not to complete full-length double-stranded cDNA synthesis nor to establish a provirus in infected non-permissive cells (Goncalves et al., 1996, Simon & Malim, 1996, von Schwedler et al., 1993). This defect could be overcome by expression of Vif *in trans* in virus producer cells but not in viral target cells, indicating that Vif function is required during late stages of virus assembly, budding and/or maturation (von Schwedler et al., 1993)(Figure 1.11). Defects of the *vif* gene do not have detectable effects on levels of viral transcription and translation in producer cells (Zhang et al., 2000a). Virions of Vif- deficient mutants are released normally from infected cells and at similar levels, and bind and penetrate target cells at the same efficiency as WT virions (Simon & Malim, 1996, von Schwedler et al., 1993). The defect appears to lie in one or more steps after virion entry, but to be determined by the presence of Vif in the producer cells.

Several reports have suggested that Vif affects the stability of the viral nucleoprotein core (Hoglund et al., 1994, Ohagen & Gabuzda, 2000, Simon & Malim, 1996). In particular, the viral nucleocapsid (NC) and reverse transcriptase were reported to be

Figure 1. 11. Vif affects viral reverse transcription

Vif deficient HIV-1 progeny virions enter target cell normally, but unlike WT HIV-1 virion, its RNA cannot be reverse transcripted to DNA. Vif may function at the late phase affecting the viral assembly/maturation.

Adapted from Inubushi & Adachi (1999)



Vif deficient HIV virion infection

less stably associated with the viral core in Δ*vif* virus particles (Ohagen & Gabuzda, 2000).

Earlier studies showed that the level of Vif within virion cores ranged from 20 to 100 molecules per virion. Other studies using highly purified virion preparations suggested that Vif is largely excluded from virions, and that the low levels observed were associated with aberrant particles containing unprocessed $Pr55^{Gag}$ (Dettenhofer & Yu, 1999, Sova et al., 2001). This suggests that mature particles may not contain Vif, and has led to the proposal that Vif is actively excluded from the virion during particle release and maturation (Dettenhofer & Yu, 1999, Sova et al., 2001). However, most recent reports suggest that Vif is incorporated into the virions (Kao et al., 2003a, Khan et al., 2001).

In addition, virion associated Vif is proteolytically cleaved, and Vif mutants with incomplete intra-virion proteolysis are incapable of supporting efficient virus replication in some cell types, suggesting that the Vif present within virions may also be important in Vif function (Khan et al., 2001)

1.2.7.3 Vif interacts with viral proteins and RNA

Subcellular fractionation studies have shown that $Pr55^{Gag}$ and Vif co-sediment in membrane-free cytoplasmic complexes (Simon et al., 1999a). Earlier work suggested that this co-sedimentation may reflect a direct interaction between Vif and the NCp7 domain of $Pr55^{Gag}$ during HIV-1 infection (Bouyac et al., 1997). However the nature of the Gag:Vif interaction remains controversial and it has been suggested rather that Gag and Vif may be independently targeted to the same subcellular compartment (Simon et al., 1999a).

There is also a reported interaction between Vif and the viral PR, which is responsible for processing of Gag and Gag-Pol precursors during viral assembly. In particular, it was reported that the N terminus of Vif (amino acids 1-98) has an inhibitory effect on HIV-1 PR. This has led to the to the proposal that Vif modulates viral assembly by regulating proteolytic cleavage of the Gag-Pol precursor (Baraz et al., 1998). Peptides derived from two regions in Vif spanning from residues 30-65 and 78-98 inhibited PR activity *in vitro*, and specifically bound HIV-PR and inhibited HIV-1 production *in vivo* (Friedler et al., 1999). Research involving peptide mapping analysis of N'-Vif revealed that Vif 88-98 was the most potent PR inhibitor. This peptide inhibited both HIV-1 and HIV-2 proteases *in vitro* (Blumenzweig et al., 2002).

Vif has also been shown to be interact with viral genomic RNA in the cytoplasm of virus producing cells (Dettenhofer et al., 2000, Zhang et al., 2000a) Zhang et.al (Zhang et al., 2000a) demonstrated that Vif specifically binds *in vitro* to HIV-1 genomic viral RNA. In the same report, the authors used co-immunoprecipitation and UV cross-linking assays to show a direct interaction between Vif and viral RNA in messenger ribonucleoprotein (mRNP) complexes in the cytoplasm of viral producing cells. Vif could be displaced from these complexes by Gag. Examination of a panel of Vif mutants indicated that there was a correlation between loss of viral RNA binding capacity and the ability to complement Vif deficient HIV-1 replication in non-permissive cells. The authors proposed a model where Vif initially binds to the NCp7 domain of the Gag precursor to mediate the engagement of HIV-1 genomic RNA to HIV-1 Gag precursors. As the viral RNA molecule binds the Gag precursor,

the affinity of Vif for RNA and Vif for Gag then decreases, and Vif gradually dissociates from the budding complex, which is now composed of Gag, Gag–Pol and genomic RNA. This displacement process of Vif might guide the proper folding and condensation during viral RNA packaging. Further, in non-permissive cells infected with Vif deficient virus, RNA could still be packaged through its interaction with Gag, but in the absence of Vif the RNA conformation might be altered, resulting in altered morphology of the virion and defects in reverse transcription (Zhang et al., 2000a). Dettenhofer *et al* also demonstrated that Vif specifically interacts with viral genomic RNA in a cell free system *in vitro* (Dettenhofer et al., 2000). During viral assembly, regions of Gag-Pol are thought to direct the specific incorporation of $tRNA_3^{Lys}$, which acts as a primer for reverse transcription. Genomic–primer tRNA complexes purified from cells infected with Vif deficient virus displayed severe defects in the initiation of reverse transcription in *in vitro* reverse transcription assays. These researchers proposed that Vif interacts transiently with RNA influencing the viral genomic RNA-$tRNA_3^{Lys}$ complex, which in turn affects reverse transcription (Dettenhofer et al., 2000). Thus, various models for Vif action involve an effect on introvirion proteolytic action, a role in particle assembly and involvement in the formation of competent genome protein/tRNA/complexes.

1.2.7.4 The effect of Vif is cell type dependent and involves cellular inhibitor(s) of HIV replication

As previously outlined, Vif is required for replication of HIV-1 in primary T-cells and monocytes/macrophages. However the requirement for Vif differs among cell lines, which are classified as non-permissive, semi-permissive or permissive for the replication of Vif-defective viruses (Sova & Volsky, 1993, Volsky et al., 1995). The conditional nature of the requirement for Vif function strongly suggests the

involvement of cellular factor(s) in Vif related functions. This led to a proposal that either permissive cells express a Vif-like function which substitutes for Vif during HIV-1 replication; or non-permissive cells express a cellular viral inhibitor which Vif is able to overcome or neutralize.

Using a cell fusion technique Simon *et al* (Simon et al., 1998a) and Madani *et al.* (Madani & Kabat, 1998) produced heterokaryons by fusing permissive and nonpermissive cells. This showed the heterokaryons had non-permissive phenotype. It has also been shown that only Vif from HIV-2 and closely related primate lentiviruses can complement a deficiency of HIV-1 Vif in human cells. Further, only HIV-1 Vif and not SIV Vif can complement an SIV Vif deletion in human cells, suggesting that Vif function is species-specific and dependent on cell type, indicating a specificity for cellular factors rather than viral factors (Simon et al., 1998b). These findings supported the proposal that non-permissive cells express an inhibitor(s) that can be overcome or neutralised by Vif during viral replication.

1.2.7.5 Host cellular factors that interact with Vif

A number of cellular factors have been shown to interact with Vif. These include:

Vimentin

Vif is predominantly present in the cytoplasm and closely colocalizes with the intermediate filament vimentin in HeLa cells, a permissive cell type (Karczewski & Strebel, 1996). It is likely that the cytoskeleton is important for virion assembly, and reverse transcription has been shown to dependent on an intact actin network (Bukrinskaya et al., 1998, Soll, 1997). Thus, an interaction of Vif with cytoskeletal

components, such as vimentin, may also contribute to its proposed function in viral assembly and correct formation of the reverse transcription complex. However co-localisation of Vif and vimentin in non-permissive cells has not been reported. Recent studies in non-permissive Cos-7 cells expressing Vif showed a dramatic effect of Vif on vimentin localisation, although Vif and vimentin did not co-localise (Henzler et al., 2001).

Hck

Hck is a tyrosine kinase from the Src family that may act as a cellular inhibitor of HIV-1 replication (Hassaine et al., 2001). In this single study Vif was demonstrated to bind specifically to the Src homology 3 domain of Hck. A glutathione S-transferase pull-down assay and mutagenesis analysis revealed that a proline rich motif (PPLP) within the C-terminal domain of Vif might be necessary for interaction with the SH3 domain of Hck. Hck was able to inhibit the production and the infectivity of Δ*vif* virus but not that of wild-type virus. Furthermore, the interaction between Vif and full-length Hck was further assessed by co-precipitation assays *in vitro* and in human cells, and overexpression of Hck in Jurkat cells rendered them less permissive. As described by Hassaine, Hck is expressed mainly in promonocytic cells, but is also present in monocyte-derived macrophages (MDM), and mediates FcγRI receptor signalling, induction of cytokine production triggered by bacterial lipopolysaccharide, phagocytosis and cell spreading (Hassaine et al., 2001). However, Hck is undetectable in primary T CD4+ lymphocytes which are non-permissive, indicating that Hck was not likely to be the primary factor determining the non-permissive phenotype of these cells to Δ*vif* virus (Hassaine et al., 2001).

HP68

HP68, a cellular ATP-binding protein described previously as an RNase L inhibitor, was shown to interact with Gag. Further, it was demonstrated to be important for post-translational events in immature HIV-1 capsid assembly (Dooher & Lingappa, 2004, Zimmerman et al., 2002), Zimmerman *et al* showed by co-immunoprecipitation that HP68 interacted with Vif in HIV-1 transfected Cos-1 cells. Consistent with previously proposed function of Vif action, this interaction might involve viral Gag to assist capsid assembly. Alternately it is possible that the association of Vif and HP68 may in some way protect viral RNA from degradation/damage during viral assembly.

Sp140

Cell factor Sp140 has also been found to interact with Vif (Madani et al., 2002). Sp140 is lymphocyte and macrophage specific and is induced by gamma interferon. The authors showed by Northern blot analysis that Sp140 mRNA was present in all nonpermissive cells studied. Confocal immunofluorescence microscopy demonstrated that Vif induced significant re-localisation of Sp140 between 16 and 20h post infection. However, co-immunoprecipitation studies exhibited a weak interaction between Sp140 and Vif. Furthermore, the authors did not examine whether Sp140 had any real effect on HIV infectivity/replication during HIV-1 infection. Thus, the biological significance of Sp140 requires further investigation.

CEM15/APOBEC3G

During the course of this study, a major new development regarding Vif action was reported by a number of laboratories (Lecossier et al., 2003, Mangeat et al., 2003,

Sheehy et al., 2002, Sheehy et al., 2003). Sheehy *et al.* identified a cellular factor designated CEM15 which is present only in non-permissive cell types. CEM15-expressing cells allowed robust replication of wild type HIV-1, but low or no detectable growth of vif-deficient HIV-1. Further the authors found that inducing transient or stable expression of CEM15 in permissive cells rendered them non-permissive, and the antiviral action function of CEM15 was overcome by the presence of Vif (Sheehy et al., 2002). Thus, CEM15 represents to date the most promising candidate for the proposed inhibitory protein determining Vif-dependent host cell restriction. CEM15 is identical to apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G, APOBEC3G. APOBEC3G is a known cytidine deaminase that introduces frequent C to U changes into the minus-strand of newly synthesised HIV-1 viral DNA during reverse transcription. When HIV replicates in cells expressing CEM15/APOBEC3G, Vif protein in the virus producer cell is able to prevent the accumulation of defects during viral DNA synthesis in newly infected target cells. A series of studies have provided convincing evidence that packaging of APOBEC3G into Vif –defective virions leads to hypermutation of viral cDNA in the target cell. Vif is thought to prevent the packaging of APOBEC3G in the progeny virions (Harris et al., 2003, Kao et al., 2003b, Mangeat et al., 2003, Mariani et al., 2003, Zhang et al., 2003). In addition, Vif also reduces APOBEC3G levels in virus–producing cells by inducing its ubiquitination and subsequent degradation by the proteasome (Marin et al., 2003, Sheehy et al., 2003, Yu et al., 2003). How Vif interferes with the packaging of APOBEC3G, and whether this function involves interactions between other cellular proteins has not been elucidated.

Our laboratory previously isolated two cDNA clones that interacted with HIV-1 Vif when expressed in the yeast 2-hybrid system. The first clone was originally named "Triad 3", as it shared 99.8% homology with the central domain of a then theoretical protein Triad 3 (AF228527) first reported by a Dutch group (Lake et al., 2003, van der Reijden et al., 1999). The second clone was named "NVBP" for Novel Vif Binding Protein with no known sequence homologue. Subsequent to publication of the complete Triad 3 nucleic acid sequences (van der Reijden et al., 1999), the corresponding protein was identified by Chen *et al* based on its biological function and the protein was named Zinc Finger protein Inhibiting NF*k*B (ZIN) (Chen et al., 2002). More recently, mRNA encoding a longer form of Triad 3 protein has also been identified and named Triad 3A (AF513717) (Chuang & Ulevitch, 2004). To avoid confusion with these more recently identified proteins our originally isolated Triad 3 peptide (Lake et al., 2003) is renamed Triad 3au. A description of the proteins and their nomenclatures are detailed in Figure 1.12. As the figure shows, Triad 3 (orange box) is the hypothetical protein reported by van der Reijden, the structure and function of which is unknown. Triad 3au is an internal segment, containing almost the whole ring finger domains of Triad 3 and ZIN protein. The relationship of these peptides and proteins, and the interactions between Triad 3au and ZIN with Vif in mammalian cells and in HIV-1 infection, were investigated in this study. More details are given in later chapters.

1.2.8 Pathogenesis of HIV-1 infection

1.2.8.1 Signs, symptoms of primary HIV-1 infection

Primary HIV-1 infection is often accompanied by signs and symptoms of an acute viral infection (Cooper et al., 1985, Kinloch-de Loes et al., 1993, Niu et al., 1993,

Figure 1.12 Nomenclature and description of Triad 3 related sequences and proteins

Upper panel:

Triad 3: The earliest description of the sequence of hypothetical ring finger protein name Triad 3 by van der Reijden et al. (van der Reijden et al., 1999), 1281bps.

Triad 3au: A partial 653 bps cDNA sequence isolated by our laboratory from a leucocyte cDNA library (Lake et al., 2003).

ZIN: The full-length sequence of Triad 3 identified by Chen et al (Chen et al., 2002), 2101 bps.

Triad3A: A mRNA recently identified by Chuang TH et al. (Chuang & Ulevitch, 2004) 2861 bps.

Bold lines indicate homologous sequence region and dashed lines represent non-homologous sequence. Red circles designate the position of stop codons, and the arrow indicates deleted sequence.

Lower panel

Orange box presents the hypothetical Triad 3 peptide sequence. Green boxes indicate ring finger domains.

ZIN: ND indicates N-domain of ZIN protein, RLDs represents ring like domain, PRD indicates the proline rich domain.

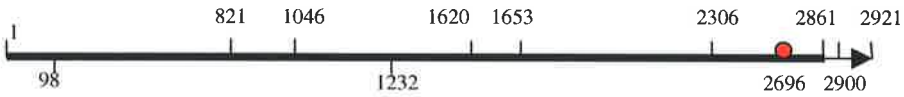
Triad 3A: published by Chuang TH et al. possessing E3 ubiquitin ligase activity.

cDNA or mRNA sequences

Triad 3(AF228527)

Triad 3au

ZIN(AY062174)



Triad 3A(AF513717)

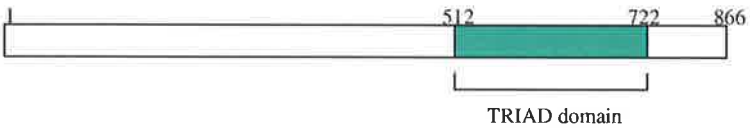
Peptide sequences and known proteins

Triad 3

Triad 3au

ZIN

Triad3A



Schacker et al., 1996, Ziegler, 1990). The symptoms usually present within days to weeks after initial exposure and infection. The most common signs and symptoms of primary HIV infection include fever, fatigue, rash, and headache. Other common signs or symptoms include lymphadenopathy; pharyngitis; myalgia; etc. (table 1.2). In contrast, infected individuals normally have none or only minor symptoms during the chronic persistent phase of HIV infection until later manifestations of HIV disease develop. Achieving the goal of eradicating HIV-1 infection from the infected host remains elusive. A greater understanding of the interaction between HIV-1 and its host may assist this work.

1.2.8.2 Aspects of virus-host interactions affecting pathogenesis

HIV-1 attaches to the CD4 surface molecule when infecting a cell. Virus entry however, is also dependent on the function of specific co-receptors. A decade following the discovery of CD4 as the attachment receptor for the HIV-1 surface glycoprotein gp120, the β-chemokine receptors CCR5 and CXCR4 were identified as coreceptors. These receptors assist HIV-1 to infect CD4+ T cells which express CXCR4 (Pleskoff et al., 1997), macrophages which express CCR5 (Gorry et al., 2001) and CD4+ dendritic cells (DCs) which express both CCR5 and CXCR4 (Xiao et al., 2000). The distribution of co-receptors on different cell types is an important determinant of virion tropism in the body.

Once HIV has integrated into the DNA of the host cell chromosome, the level of expression of the provirus is dependent on the activation state of the host cell. Provirus transcription needs host factors that are abundant in activated T cells and macrophages. These factors, together with the viral Tat protein, markedly promote

Table 1.2

Signs, Symptoms and Laboratory Values of Primary HIV Infection

Signs, Symptoms, Laboratory Values	Frequency (%)
Fevers	>90%
Fatigue	>90%
Rash	>70%
Headache	32-70%
Lymphadenopathy	40-70%
Pharyngitis	50-70%
Myalgia, arthralgia	50-70%
Nausea, vomiting or diarrhea	30-60%
Night sweats	50%
Oral ulcers	10-20%
Genital ulcers	5-15%
Thrombocytopenia	45%
Leukopenia	40%
Elevated hepatic enzymes	21%

Adapted from Http://hivinsite.ucsf.edu

gene expression, leading to virion production, antigen display and a range of deleterious outcomes for the cell related to virion replication. Insert here known reasons for the decline in T cell numbers in HIV-1 infected patients related to virus replication consequences eg apoptosis, etc. In contrast, quiescent T cells are refractory to infection because of blocks at or immediately after reverse transcription. These blocks prevent the establishment of the provirus, thereby preventing later stages of virus replication and immune clearance of infected cells. However certain combinations of specific cytokines, including IL-2, IL-4, IL-7, or IL-15 can activate quiescent cells and hence trigger viral replication in these cells (Unutmaz et al., 1999). Viral factors may also be involved in promoting HIV-1 infection of quiescent host cells. For example, Nef has been shown to create conditions that allow virus to infect non-activated cells. HIV-1 infected macrophages also release soluble factors that stimulate activation of T cells leading to the infection with wild-type but not HIV Δ*nef* virions (Swingler et al., 2003).

Cells of the macrophage lineage play an important role in initial infection with HIV-1 and contribute to the pathogenesis of the disease throughout the course of infection. Both blood monocytes and tissue macrophages can be infected with HIV-1 *in vivo* and *in vitro*, although macrophages are more susceptible to infection via the CD4 receptor and CCR5 co-receptor. Cells of the macrophage lineage can be infected predominantly with strains that preferentially use the CCR5 co-receptor and are designated R5 or macrophage (M)-tropic strains, although infection with some T cell line (T)-tropic (X4) strains or dual-tropic isolates of HIV-1 (exhibiting features of both M-tropic and T-tropic isolates) has also been reported (Kedzierska & Crowe, 2002). Macrophages are a second major reservoir of HIV-1 infection *in vivo*. The

dendritic cell (DC) reservoir is the least well understood of HIV-1 infected cell targets. It has been generally presumed that DCs function as a scavenger for virions at the site of transmission in the mucosa, and these cells may be one of the initial target cells for HIV-1 infection. There is now growing evidence to suggest that certain DCs also support virus replication and migrate to the lymphoid tissue where they can contribute to the dissemination of the virus to adjacent CD4+ T cells (Lore & Larsson, 2003, Piguet & Blauvelt, 2002)

1.2.8.3 Developments in viral pathogenicity

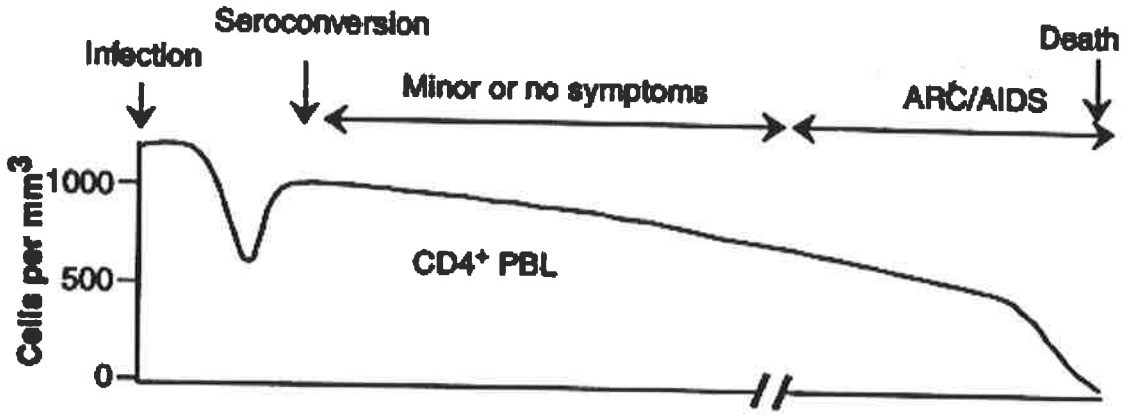
Measurement of virus load and CD4+ lymphocyte levels in HIV infected patients are important markers of HIV-1 progression (Ho et al., 1995, Wei et al., 1995). The steady-state level of viremia "set point" that is reached after acute infection can be predictive of the ultimate rate of clinical progression (Mellors et al., 1996). Others have demonstrated a causal relationship between the viral replication rate and lymphocyte depletion with disease progression (Alimonti et al., 2003, Deacon et al., 1995) (Figure 1.13). However, it is not clear whether CD4 T lymphocyte depletion, which steadily occurs during the asymptomatic phase of the infection, is a consequence of direct killing of infected cells by viral replication or an indirect consequence of viral infection, and arguments actually support both mechanisms. For example, CD4+ and CD8+ T cells from individuals infected with HIV-1 have been found to undergo spontaneous apoptosis *in vitro* (Meyaard et al., 1992). However, analysis of lymph nodes infected with HIV-1 using in situ labelling of lymph nodes from HIV-infected children and SIV-infected macaques has shown that HIV-1 replication in these tissues cause cell death of infected cells and apoptosis of uninfected bystander cells (Finkel et al., 1995). Several mechanisms for CD4 T

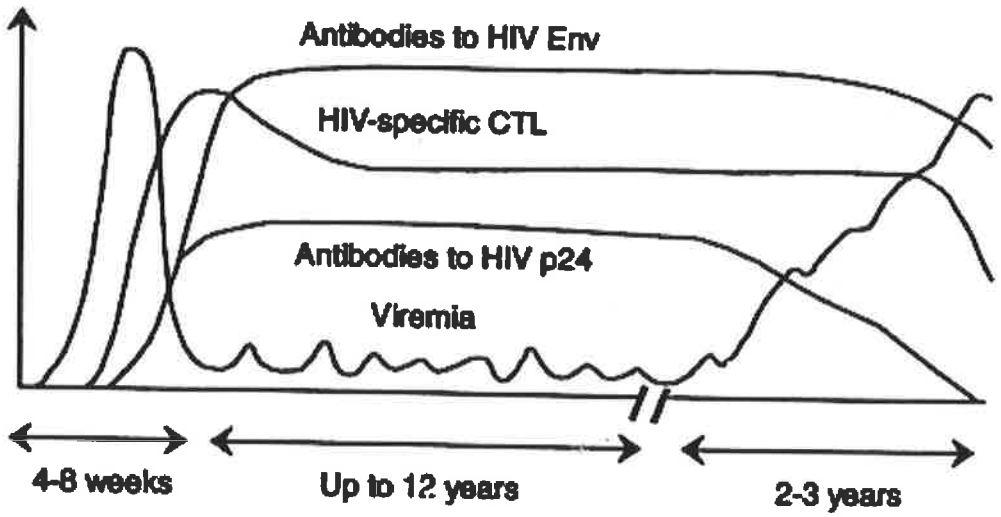
Figure 1.13 Time course of HIV-1 infection

Upper panel: Decline of CD4+ lymphocytes in peripheral blood, in relation to the three stage of disease; primary infection that precedes seroconversion, a long asymptomatic period, and late development of symptoms (known as the AIDS-related complex, ARC) and AIDS.

Lower panel: Course of development and decline of viraemia, antibodies specific for Env and p24 and HIV-specific cytotoxic T lymphocytes (CTL)

Adapted from Weiss (1993)


Seroconversion
Infection
Death
Minor or no symptoms
ARC/AIDS
Cells per mm3
1000
500
0
CD4+ PBL


Antibodies to HIV Env
HIV-specific CTL
Antibodies to HIV p24
Viremia
4-8 weeks
Up to 12 years
2-3 years

lymphocyte depletion in HIV infection have also been proposed. Earlier studies demonstrated that although CD4 expression is essential for HIV induction of apoptosis by the HIV envelope, the apoptotic signal could be delivered in the absence of the CD4 cytoplasmic domain, suggesting that other membrane associated molecule(s) are recruited for the signalling to initiate apoptosis (Jacotot et al., 1997). Daniel V *et al* showed evidence supporting the contention that autoantibody-induced apoptotic and non-apoptotic mechanisms contribute to CD4 depletion in long-term HIV-infected haemophilia patients (Daniel et al., 2004). By investigating the ability of HIV to trigger dendritic cells (DCs) cytotoxicity, Lichtner M. *et al* showed that HIV-1-infected DCs induce apoptotic death in infected and uninfected primary CD4 T lymphocytes. When incubated with HIV, monocyte-derived DCs induced apoptosis in MDA-231 cells, which are sensitive to MV (measles virus) -induced DC cytotoxicity, and in uninfected as well as HIV-infected H9 CD4+ T cell lines (Lichtner et al., 2004).

HIV-infected humans and SIV-infected rhesus macaques who remain healthy despite long-term infection exhibit exceptionally low levels of virus replication and active antiviral cellular immune responses (Cao et al., 1995, Migueles & Connors, 2002, Rosenberg et al., 1997). In contrast, sooty mangabey monkeys that represent natural hosts for SIV infection do not develop AIDS despite high levels of virus replication and limited antiviral CD8 (+) T cell responses (Broussard et al., 2001, Chakrabarti et al., 2000, Rey-Cuille et al., 1998). Similar studies by Silvestri *et al* indicated that SIV-infected mangabeys preserve T lymphocyte populations and regenerative capacity and manifest far lower levels of aberrant immune activation and apoptosis than are characteristic of pathogenic primate lentiviral infections (Silvestri et al.,

2003). These results suggest the direct consequences of virus replication alone can not account for progressive $CD4^+$ T cell depletion leading to AIDS. CD4+ responses, CD8+ T cell activity, and also antibodies and other factors may contribute to control of the AIDS development without antiretroviral therapy in some HIV-positive individuals, but immune activation may also contribute to development of disease.

1.3 HIV/AIDS vaccine developments

Since the discovery of HIV, great progress has been made in the molecular virology, pathogenesis and epidemiology of HIV, but the development of an effective HIV vaccine remains an elusive goal. A number of reasons may explain the slow progress in vaccine development. These include: **1**) Integration of the virus into the host cell genome making virus eradication more difficult; **2**) Infection of long-lived immune cells; **3**) HIV genetic diversity (especially in its envelope); **4**) Persistent high viral replication releasing up to 10 billion viral particles per day, and/ or production of immunosuppressive products or proteins; **5**) The lack of readily accessible small animal models; **6**) The lack of a clear naturally occurring state of immunity to challenge, that could provide a model. In addition, traditional neutralising antibody is sufficient for some vaccines, but vaccines aimed at stimulating neutralising antibodies were unable to prevent HIV infection.

Generally, live attenuated vaccines are considered more effective than inactivated virus or purified protein subunit vaccines, because live attenuated vaccines have better ability at stimulating CD8 T-cells than inactive immunogens. Currently more than 20 HIV vaccines designed to stimulate T-cell responses are being developed.

These are based on the idea that induction of strong CD8 and CD4 T-cell responses through vaccination will abort or control early HIV infection after challenge. Cytotoxic CD8 T cells kill infected cells and hence halt production of virus. CD8 cells may also inhibit entry of HIV-1 by releasing the β-chemokines RANTES, MIP-1α and MIP-1 β, which compete for the CCR5 receptor (McMichael & Hanke, 2003), and other cytokines with antiviral activity. In studies of rare individuals who are highly exposed to HIV but remain uninfected for prolonged periods of time, strong CD8 T-cell responses have been observed. Such individuals can also have CD4 T-cell responses to HIV but no serum antibodies (McMichael & Hanke, 2003). This lends support to the idea that vaccination that leads to a strong CD8 T-cell response may prevent or abort early infection in humans (Kaul et al., 2001, Rowland-Jones et al., 1998).

Some recent vaccines designed to stimulate CD8 and CD4 T-cell immunity have shown promising results in animal models (Amara et al., 2001, Barouch et al., 2000, Belyakov et al., 2001, Harvey et al., 2003, Shiver et al., 2002). Thus, HIV/AIDS vaccine development is a long-term and very challenging campaign, which needs further major efforts from the research communities.

1.4 HIV/AIDS treatment

Compared with HIV/AIDS vaccines, treatment of HIV infection has made significant more progress. When AIDS was first recognised in 1981, patients did not often live longer than one or two years following the onset of AIDS. A number of antiviral drugs, which specifically interrupt viral replication, have since been developed, which allow infected individuals to live longer and healthier. These drugs include

inhibitors targeting essential steps of HIV-1 replication including virus entry, reverse transcription, viral protein cleavage, and more recently integration.

Reverse transcriptase inhibitors

Available reverse transcription inhibitors act by specifically targeting the function of RT and fall into two groups: nucleoside and non-nucleoside inhibitors. Nucleoside inhibitors (NRTIs) which result in premature termination of reverse transcription include Abacavir (Ziagen), Zalcitabine (ddC), Didanosine (ddI, Videx), Stavudine (d4T, Zerit), Lamivudine (3TC, Epivir), Zidovudine (ZDV), Tenofovir (Viread). In contrast, non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to RT and block its enzymatic functions by inducing conformational or other steric effects on the enzyme active site. These agents include Delavirdine (Rescriptor), Nevirapine (Viramune) and Efavirenz (Sustiva, Stocrin). Although reverse transcription inhibitors form the formulation for antiviral treatment in HIV, virus isolates soon emerge with resistance to these drugs in patients undergoing treatment. Therefore patients are treated with a combination of drugs also targeting other viral proteins as discussed below. This further emphasises the importance for continued research of other candidate antiviral targets.

Protease inhibitors

The viral protease is essential for cleavage of the Gag and Gag-Pol polyprotein precursors during virion maturation. Protease inhibitors are designed to block the viral enzyme and thereby inhibit these normal critical functions during HIV-1 replication. Studies have shown protease inhibitor can reduce the amount of virus in the blood and increase CD4 cell counts (Lim et al., 2003). Food and Drug

Administration (FDA) approved protease inhibitors include Amprenavir (Agenerase), Indinavir (Crixivan), Lopinavir/Ritonavir (Kaletra), Ritonavir (Norvir), Saquinavir (Fortovase), Nelfinavir (Viracept), and Atazanavir (Reyataz). Therapy involving the combination of RT inhibitors and protease inhibitors, or therapy based on dual NRTI and an NNRTI combination, is known as Highly Active Anti-Retroviral Therapy, HAART.

Other inhibitors

Despite the success of HAART in improving the clinical prognosis of HIV-infected patients, therapeutic options are limited for many patients, particularly those with extensive treatment experience. Resistance, cross-resistance, and toxicity all contribute to impairing the long-term efficacy of current antiviral drugs, necessitating the development of novel agents. The HIV replication cycle has many processes that are potential targets for inhibition in addition to the current reverse transcriptase and protease targets. These include HIV entry, HIV integrase, nucleocapsid assembly, and Tat proteins. Entry inhibitors can be classified as attachment, co-receptor, and fusion inhibitors according to the stage in the entry process at which they act. Most advanced in development of these is the fusion inhibitor enfuvirtide (Ball & Kinchelow, 2003, Burton, 2003, Fletcher, 2003, O'Brien, 2003).

Other problems with current HIV drug treatment regimes do occur, apart from long-term resistance of HIV to antivirals. These include the current failure to totally eradicate virus - infected cells containing integrated proviral genomes with any current forms of therapy. Another problem compounding this is poor patient compliance to the treatment regime permitting escape and virus production from

these stable virus reservoirs. Finally, other problems include unwanted side effects to drugs and the cost of treatment. Thus, these issues contribute to a situation where HIV-1 infection is still one of the leading infective causes of human fatality worldwide.

1.5 Summary

AIDS was first officially detected in United Stated in 1981. HIV-1, the causative agent, is a complex retrovirus which contains the structural and enzymatic proteins Gag, Pol, and Env, and six accessory proteins including Tat and Rev (also called regulatory proteins) and Vif. Although the mechanism of HIV infection in some human cell types, such as microglial, dendritic and Langerhans cells, has not been fully investigated, the process of HIV replication in CD4 T cells is relatively well described. When HIV infects a CD4 T cell the RNA genome is reverse transcribed to DNA in the cytoplasm of the cell, the newly synthesised DNA then enters the nucleus and is integrated into host cellular DNA. Under the regulation of cellular factor(s), such as NF-kappa B, and viral proteins including Tat and Rev, the HIV genome is transcribed and translated proteins assemble to form mature virions. HIV infection of human cells leads to CD4 T cell depletion in the host, eventually leading to the development of AIDS. The use of highly active antiretroviral therapy (HAART) has made it possible to reduce the plasma viral load of most infected individuals to undetectable levels and slow progression of this disease. However despite successful control of plasma viremia in HIV-infected individuals undergoing HAART, HIV-1 DNA continues to persist in peripheral blood mononuclear cells (PBMC) and accompanied by low level viral replication in lymphoid tissues (Cavert et al., 1997, Perelson et al., 1997). Moreover, drug resistant strains inevitably emerge

during HAART. In the absence of a suitable vaccine for HIV-1 infection, HIV is still a major infective cause of human fatality worldwide. In the past, treatment of HIV-1 infection has mainly focused on triple drug combinations including inhibitors of reverse transcriptase and protease (Chun et al., 1997). It is clear that more pathways for drug intervention must be found.

HIV viral infectivity factor, Vif is the most conserved viral accessory protein in HIV and other lentiviruses, such as FIV and SIV. Experiments *in vitro* using numerous laboratory cell lines have shown that Vif is crucial for viral replication and infectivity in some cell types but not others (Aldrovandi & Zack, 1996, Gabuzda et al., 1992). Recent research has shown that Vif overcomes a cellular inhibitor (APOBEC3G) that is present in nonpermissive cell types which predominate *in vivo*. Further studies demonstrated Vif could prevent CEM15 packaging and cause CEM15 degradation, but the mechanisms for these actions have not been fully elucidated. Vif is also known to interact with a number of other host cellular proteins but the roles of these additional interactions in the action of Vif are not known. Discovering the molecular mechanism(s) underlying the role of these cellular proteins in Vif function will be of great value in further understanding HIV-1 replication and virus pathogenesis, and in identifying potential new targets for therapeutic intervention and possible vaccines strategy.

1.6 Hypotheses

1) There may be cellular factor(s) in addition to CEM15/APOBEC3G in human cells that interact with HIV-1 Vif and are involved in Vif function;
2) These factor(s) may affect HIV-1 infectivity and /or replication

1.7 Specific aims

Our laboratory previously isolated two partial cDNA clones from a human leucocyte cDNA library that interacted with HIV-1 Vif by using the Yeast 2-hybrid system. These two peptide sequences were named novel Vif interacting protein, "NVBP" and "Triad 3" (Lake et al., 2003). Since the more recent discovery of other Triad 3 – like proteins with similar nomenclature, the original partial cDNA clone is referred to as Triad 3au in this study. In this study the interaction between Vif and Triad 3au and/or its protein homologue ZIN were examined in mammalian cells, and the biological relevance of such interactions were investigated. The specific aims of this thesis are:

1) To investigate whether NVBP and Triad 3au interact with Vif in human cells
2) To investigate whether the cellular protein homologue of Triad 3au, ZIN also interacts with Vif in human cells
3) To examine the cellular distribution of Vif, ZIN and transfected Triad 3au in mammalian cells
4) To determine what affect an interaction between Triad 3au and/or ZIN with Vif may have on HIV- infection
5) To discuss the role of an interaction between Vif and ZIN in HIV-1 infection in the context of known interactions with other cell proteins.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cells and cell culture

HeLa, 293T and Cos-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM); and H9, SupT1, CEM-ss, A3.01 and HuT-78 cells in RPMI-1640 medium. Both media were supplemented with 10% fetal calf serum, 2 mM L-glutamine and 1.2 μg/ml penicillin and 16 μg/ml gentamycin. HeLa-CD4-LTR-β-gal cells were cultured in complete DMEM medium with the addition of G418 and Hygromycin B at final concentrations of 200 μg/ml and 100 μg/ml (medium A) respectively. To prepare peripheral blood lymphoctyes (PBL) and macrophages, whole blood was collected and peripheral blood mononuclear cells (PBMC) were isolated using lymphoprep™ (Vital Diagnostics). Briefly, 45 mls of fresh blood was brought to 100 mls with Hanks medium, mixed and divided to four 50 mls of polypropylene conical tubes (25 mls each tube). Next, 20 mls of lymphoprep was added slowly to the bottom of each tube. These were then subjected to centrifugation at 4°C 2,000 rpm 20 min in J6 centrifuge (BECKMAN). The white cell layer was taken out carefully, added to a fresh tube and brought to 50 mls with Hanks. Using the same centrifuge, these samples were spun again but at 4°C 1,000 rpm 5 min. The supernatants were removed and the cells were washed with Hanks, and spun to discard the Hanks. Subsequently, cells of monocytes/macrophage lineage were separated from peripheral blood lymphoctyes (PBL) by their adherence to plastic. The contamination

in the described method has been found to be 1-5 per cent of the total cell number (Harris et al.,1969, Thorsby et al.,1970)

All cell lines were cultured at 37°C in an atmosphere containing 5% CO_2. For cell splitting, adherent cells such as 293T cells and HeLa CD4 cells were split when grown full confluent using conventional trypsin treatment method. Confluent cells were washed with 0.01M EDTA PBS and treated with Trypsin 2-3 min, the cells were then collected when suspended by a centrifugation at 1000 rpm 25 °C 5 min in GPR centrifuge (BECKMAN). Next, the cells were suspended in culture medium and divided to fresh flasks. For suspension cells such as HuT78 cells and CEM-ss cells, cultures were split when grown to high density. These suspension cells were collected and split using the same method described above.

2.1.2 Bacterial culture

Escherichia coli (E.coli) strain DH5α was propagated in 1x LB medium or on plates containing 1x LB and 15g/L bacto-agar at 37°C for 12-18 hr. Transformed DH5α was grown in 1x LB containing 100 μg/ml ampicillin (Boehringer Mannheim) or on 1x LB and 15g/L bacto-agar plates containing 100 μg/ml ampicillin. Bacterial cultures were stored in LB containing 15% glycerol at -70 °C.

Competent DH5α was prepared by growing DH5α to log phase growth (OD_{600} ~ 0.6) in 50 mls LB broth. Cells were pelleted at 6000 rpm in JA-20 rotor (J2-21M/E centrifuge, BECKMAN) for 10 min at 4 °C and suspended in 25 mls ice-cold 0.1 M $MgCl_2$ followed by the same centrifugation condition as above. The pellet was resuspended in 12.5 mls ice-cold 0.1M $CaCl_2$ and incubated on ice for 20 min. Cells

were then collected using the same centrifugation method and resuspended in 1.6 mls ice-cold transformation buffer (10 mls 1M $CaCl_2$, 20 mls 80% glycerol, 70 mls H_2O). Cell slurry was aliquoted in 200 µl volumes and stored at -70 °C.

Compared to DH5α cells, *Escherichia coli (E.coli)* BL_{21} cells are deficient in the OmpT and Lon proteases, which may interfere with isolation of intact recombinant proteins. They are designed for high-level protein expression using T_7 RNA polymerase-based expression systems. The BL_{21} used in this study were taken from the bacteria stock available in HIV laboratory of IMVS and grown under the same culture conditions as DH5α cells.

2.1.3 Yeast AH109 and cell culture

Yeast AH109 was purchased from Clontech. The parental AH109 cells were cultured in Minimal SD Base and Minimal SD Agar Base, either with dextrose (glucose) or galactose + raffinose (Cat # 8603-1,Clontech). For checking transfection efficiency, AH109 cells transfected with different combination of pACT2, pGBKT7, pACT2-Triad 3au, pACT2-NVBP, and pGBKT7-Vif were cultured in SD/-Leu/-Trp. To test for the interaction between Vif and Triad 3au or NVBP in yeast, AH109 cells containing both constructs were cultured in SD/Leu/Trp/Ade/His/X-gal (also see the section 2.2.1). All these media recipes were adapted from Yeast Protocols Handbook provided by the same company (protocol # PT3024-1, Clontech).

2.1.4 Plasmid vectors

pGBKT7 (Cat # K1612-1) and pACT2 (Cat # K1604-A) used for yeast two-hybrid assay were both purchased from the Clontech. The bait vector pGBKT7 carries the

Kan^r marker for selection in *E.coli* and the *TRP1* nutritional marker for selection in yeast (Figure 2.1 A). The prey vector pACT2 is a shuttle vector that replicates autonomously in both *E.coli* and S.cerevisiae and carries the bla gene, which confers ampicillin resistance in *E.coli*. pACT2 also contains the *LEU2* nutritional gene that allows yeast auxotrophs to grow on limiting synthetic media (Figure 2.1B). pCMV-HA (Cat # K6003-1) and pCMV-Myc (Cat # K6003-1) used in co-immunoprecipitation experiments were also bought from Clontech (Figure 2.2). These vectors are similar with the exception of the epitope they express. The expression vector p3XFLAG-CMV-^{TM}-10 (Sigma, product No. E4401) was a gift from Dr. Pinghui Feng (Harvard Medical Center). This vector encodes three adjacent FLAG epitopes (Asp-Tyr-Lys-Xaa-Xaa-Asp) upstream of the multiple cloning region (Figure 2.3). pGEX 2T was purchased from Amersham Biosciences (Figure 2.4.) and used in GST-Vif fusion protein expression for the GST pull-down assay.

Both Small-scale DNA and Large-scale preparation of plasmid DNA from bacteria were performed using Qiagen Plasmid Extraction Kits (Cat #s: 27104 and 12163).

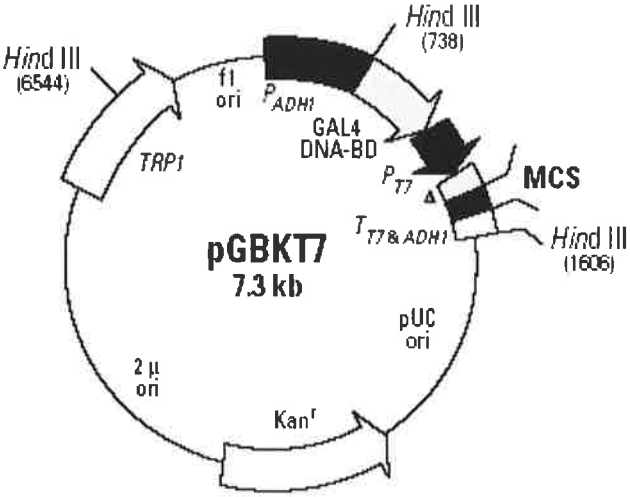
2.1.5 Oligonucleotide sequences

All oligonucleotide sequences used in this project were synthesized by GeneWorks. Since the different experiments were performed using different experiment systems, NVBP, Triad 3au or ZIN and Vif were inserted into different vectors (details described in later chapters). Oligonucleotide pellets were resuspended at the required concentration in sterile water. The sequences and coordinates of the oligonucleotide primers used in this study are listed in Table 2.1. Underlined sequences represent enzyme digestion sites allowing the PCR products to be subcloned into relevant vectors after treatment with restriction enzymes.

Figure 2.1 Vector information of pGBKT7 (A) and pACT2 (B)

Adapted from Clotech website http://www.bdbiosciences.com/clontech/

A



▲ c-Myc epitope tag

B

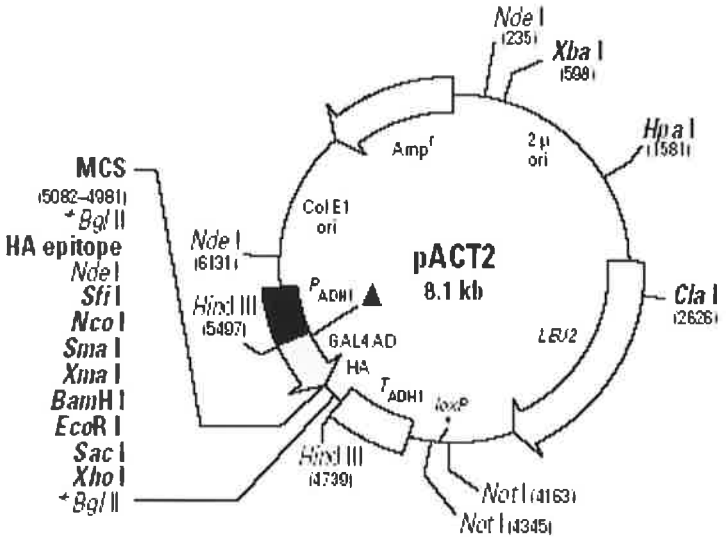
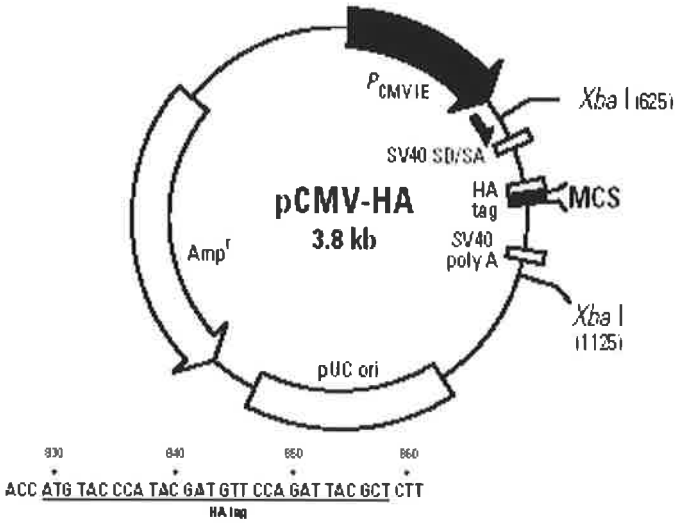


Figure 2.2 Vector information of pCMV-HA (A) and pCMV-Myc (B)

Adapted from Clotech website http://www.bdbiosciences.com/clontech/

A


P CMV IE
Xba I (625)
SV40 SD/SA
pCMV-HA
3.8 kb
HA tag
MCS
Amp r
SV40 poly A
Xba I (1125)
pUC ori
830
840
850
860
ACC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT CTT
HA tag

B

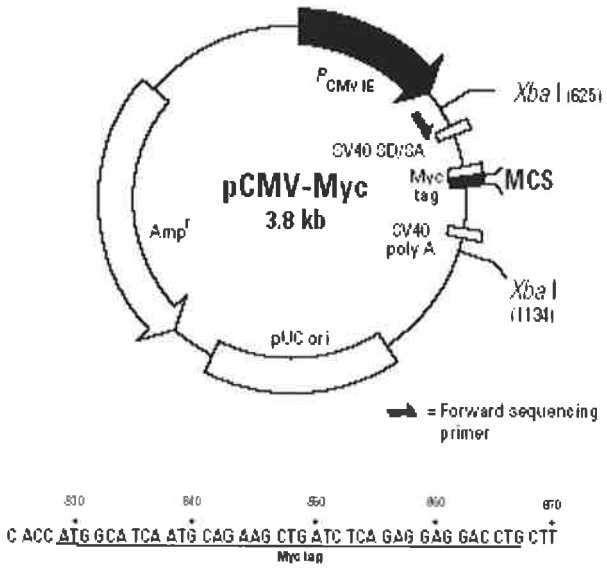
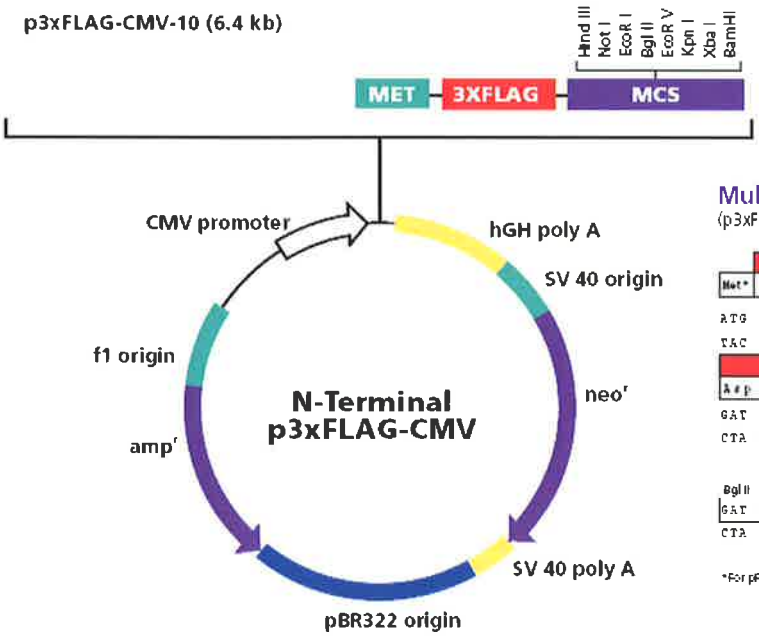

P CMV IE
Xba I (625)
SV40 SD/SA
pCMV-Myc
3.8 kb
Myc tag
MCS
Amp r
SV40 poly A
Xba I (1134)
pUC ori
= Forward sequencing primer
830
840
850
860
870
C ACC ATG GCA TCA ATG CAG AAG CTG ATC TCA GAG GAG GAC CTG CTT
Myc tag

Figure 2.3 Vector information of p3XFLAG-CMV-10

Adapted from sigma website http://www.sigmaaldrich.com



Multiple Cloning Site

(p3xFLAG-CMV-9* and p3xFLAG-CMV-10)

3XFLAG Peptide Sequence

Met* Asp Tyr Lys Asp His Asp Gly Asp Tyr Lys Asp His Asp Ile

ATG GAC TAC AAA GAC CAT GAC GGT GAT TAT AAA GAT CAT GAC ATC

TAC CTG ATG TTT CTG GTA CTG CCA CTA ATA TTT CTA GTA CTG TAG

3XFLAG Peptide Sequence

Asp Tyr Lys Asp Asp Asp Asp Lys

GAT TAC AAG GAT GAC GAT GAC AAG CTT GCG GCC GCG AAT TCA TCG ATA

CTA ATG TTC CTA CTG CTA CTG TTC GAA CGC CGG CGC TTA AGT AGC TAT

Hind III NotI EcoRI

BglII EcoRV KpnI XbaI BamHI

GAT CTG ATA TCG GTA CCA GTC GAC TCT AGA GGA TCC CGG TG

CTA GAC TAT AGC CAT GGT CAG CTG AGA TCT CCT AGG CCC AC

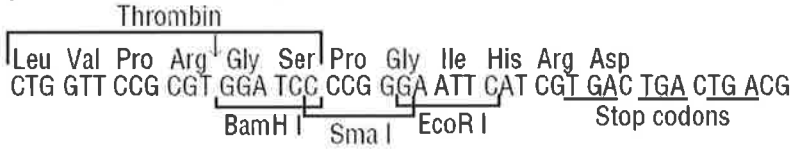
*For pFLAG-CMV-9, the Met-preprotrypsin leader sequence (PPT LS) precedes the FLAG coding sequence.

Figure 2.4 pGEX-2T vector information

Map of the glutathione S-transferase fusion vector showing the reading frame and main endonuclease restriction enzyme sites

Adapted from website http://www5.amershambiosciences.com

pGEX-2T (27-4801-01)



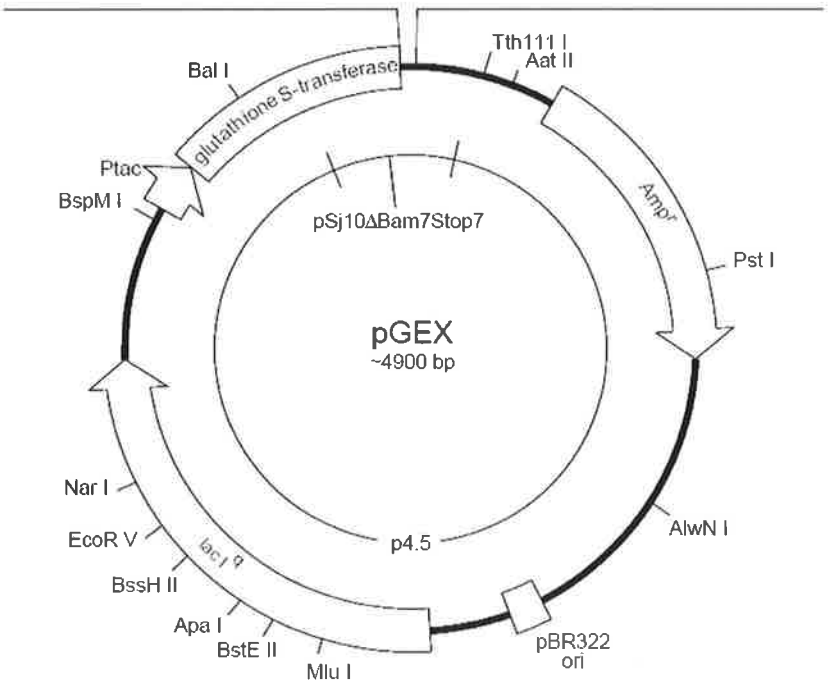


Table 2.1 Oligonucleotide sequences of primers used in this study

Name	Sequence	Region specific
Vif Ea(+)	5'-cacacacgaattcatggaaaacagatggca-3'	Vif in pGBKT7 vector
Vif Eb(+)/ Vif DS(A)	5'-caccgaattcggatggaaaacagatggc-3'	Vif in pCMV-Myc vector
Vif S(-)/ Vif DS(D)	5'-cacacacgtcgacctagtgtccattcattg-3'	Vif in pGBKT7 / pCMV-Myc vector
Vif B(+)	5'-cacacacggatccatggaaaacagatggca-3'	Vif in pGEX-2T vector
Vif E (-)	5'-cacacacgaattcctagtgtccattcattg-3'-3'	Vif in pGEX-2T vector
TrE(+)	5'-cacacacgaattcgaggggaatttccattcgag- 3'	Triad 3au in pCMV-HA vector
TrX(-)	5'-cacacacctcgagagtgggatcggtccagagag- 3'	Triad 3au in pCMV-HA vector
ZnE(+)	5'-cacacacgaattctatggccgacttcaaagtgc-3'	ZIN in p3XFLAG-CMV-10
ZnB(-)	5'-cacacacggatcctcagaagcgatgccgcggct-3'	ZIN in p3XFLAG-CMV-10
SS(+)	5'-ctaactagggaacccactgc-3'	NL4.3 strong stop
SS(-)	5'-gggtctgagggatctctagt-3'	NL4.3 strong stop
NL4(+)	5'-agatctctcgacgcaggact-3'	NL4.3 gag
NL4(-)	5'-tctctctccttctagcctcc-3'	NL4.3 gag
pCMV(+)	5'-gatccggtactagaggaactgaaaaac-3'	5' end of the inserts in pCMV vectors
pCMV(-)	5'-acttgtttattgcagcttataatggtt-3'	3' end of the inserts in pCMV vectors
CMV30	5'-aatgtcgtaataaccccgccccgttgacgc-3'	5'end of the inserts in p3XFLAG-CMV-10
Zn1a (+)	5'-atatgcccaagaggcagtctttggatc-3'	ZIN later part
MM5'	5'-ctattcgatgatgaagataccccaccaaaccc-3'	5' end of the inserts in pACT2 vector
MM3'	5'-aagtgaacttgcggggtttttcagtatctacg-3'	3' end of the inserts in pACT2 vector
Vif DS(B)	5'-tactaatttagtactatcatcccctagtgggat-3'	Vif mutant DS insertion area
Vif DS(C)	5'-actaggggatgatagtactaaattagtaataac-3'	Vif mutant DS insertion area
NvE(+)	5'-cacacacgaattcgacttgcacagcagctgcac-3'	5'end of NVBP in pCMV
NvX(-)	5'-cacacacctcgagtcaatcacgagggaccgaga-3'	3'end of NVBP in pCMV
3'-CDS primer A	5'-aagcagtggtatcaacgcagagtac(t)30vn-3 (n = a,c,g or t; v = a,g, or c	Poly A region of mRNA

2.1.6 Antibodies

HA–Tag polyclonal antibody (Cat # 3808-1) was purchased from Clontech. Mouse anti-c-Myc is a product of ZYMED LABORATORYES INC, Cat # is 13-2500, clone #9E10. The Myc-Tag 9B11(Cat # 2276) monoclonal antibody and HA-Tag 262K(Cat # 2362) monoclonal antibody were free samples provided by Cell Signaling Company. Anti-FLAG M2 Monoclonal Antibody was purchased from Sigma (Product Code F3165). Anti-GST-HRP Conjugate was ordered from Amersham Pharmacia Biotech (Product code RPN1236). The secondary antibodies conjugated with peroxidase for use in western blots, (anti-rabbit IgG, (H+L) (Product #31460) and anti-mouse IgG, (H+L) (Product #31430)), were purchased from PIERCE company. The secondary antibodies for confocal microscopy, Alex Fluor 488 goat anti-rabbit IgG (H+L) (Cat # A-11008) and Alex Fluor 546 goat anti-mouse IgG (H+L) (Cat # A-11003), were purchased from Molecular Probes company. All antibodies were used at concentrations recommended by the manufacturer or at optimized concentrations determined in preliminary experiments.

2.1.7 Commonly used buffers and solutions

DNase buffer (5x):

0.25 M Tris-HCl pH 7.5, 35 mM $MgCl_2$, 2.5 mM DTT

Ethidium bromide stock solution:

10 mg/ml ethidium bromide (Sigma) dissolved in DDW to reach a concentration 4 µg/ml and store at 4 °C in a dark bottle.

LB (1x):

10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl

PBS:

140 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4

SSC (1x)

150 mM NaCl, 15 mM trisodium citrate, pH 7.0

TAE (1x):

40 mM Tris-acetate, 1 mM EDTA

TBE (1x):

90 mM Tris, 90 mM Boric acid and 2.4 mM disodium EDTA

SDS gel loading buffer (6x):

0.35 M Tris-HCl (pH 6.8)

10.28% (w/v) SDS

36% (v/v) Glycerol

5% β-mercaptoethanol

0.012% bromphenol blue

RNA loading buffer (5x):

16 µl saturated aqueous bromophenol blue solution

80 µl 500 mM EDTA, pH 8.0

720 µl 37%(12.3 M) formaldehyde

2 ml 100% glycerol

3.084 ml formamide

4 ml 10x FA gel buffer

RNase-free water to 10 ml

TBS (1x):

50 mM Tris pH 7.4

135 mM NaCl

TBST (1x):

TBS+0.1% Tween-20

Transfer Buffer (1x):

48 mM Tris

39 mM Glycine

SDS (0.0375% w/v)

20% Methanol

Running buffer (5x):

Tris	15 g/L pH 8.3
Glycine	72 g/L
SDS	5 g/L

HIRT Solution 1 (1x):

5 mM Tris pH 7.7; 10 mM EDTA

HIRT Solution 2 (1x):

10 mM EDTA; 5 mM Tris pH 7.7; 1.2% SDS

Formaldehyde agarose (FA) buffer (10x):

200 mM 3-[N-Morpholino] propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

FA gel running buffer (1x):

100 ml 10x FA gel buffer

20 ml 37%(12.3M) formaldehyde

880 ml RNase-free water

2.2 Methods

2.2.1 Investigation of Vif/Triad 3au or NVBP interactions in yeast

Detailed procedures for using the yeast two-hybrid system were provided by Clontech. Briefly, yeast strain AH109, which has the *ADE2*, *HIS3* and *lacZ* (or *MEL1*) reporter genes under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes (Figure 3.3), was used as the recipient host. To improve the transformation efficiency, sequential transformations were performed. The yeast strain AH109 was first transformed with the bait plasmid pGBKT7-Vif and recipient cells were selected by growth on tryptophan (Trp)-deficient synthetic dropout (SD) media. Cells harboring this bait plasmid were checked for self-activation on SD media lacking Trp. Yeast cells pre-transformed with bait plasmid were then transformed with the isolated plasmids pACT2-Triad 3au or pACT2-

NVBP and transformants were selected on SD medium lacking Trp and Leucine (Leu). Grown clones then were streaked onto SD medium lacking Adenine (Ade), Histidine (His), Leu, Trp. Clones, which could grow on Ade, His, Leu, Trp deficient SD medium were screened by α-gal, a substrate for *lacZ* encoding beta-galactosidase. Blue clones were isolated and their plasmids were prepared and analysed by DNA sequencing (MM5' and MM3' primers) (see table **2.1**). Both strands of an insert were sequenced. Once sequences of library isolates were obtained, homology searches for the DNA sequences were performed at the National Center for Biotechnology Information with the BLAST network service.

2.2.2 Production of Triad 3au DNA probe

The pHA-Triad 3au construct was subjected to double restriction enzyme digestion and agarose gel electrophoresis. The Triad 3au specific band was extracted using QIAquick Gel Extraction Kit (Qiagen Cat #: 28704) following the direction of the manufacturer. The concentration of the Triad 3au DNA was determined by using an Ultra spec 3000 Spectrophotometer (Pharmacia Biotech). The Megaprime Kit was employed for DNA labelling. Briefly, 100 ng of Triad 3au DNA was diluted in 5 µl dH_2O, 5 µl Megaprime primer solution was added and the mixture heated to 100 °C for 5 min. The reaction was spun briefly and allowed to cool to room temperature. Next, 16µl of dH_2O was added, followed by 4 µl of each non-labelled nucleotide: dGTP, dTTP and dCTP; 5µl of reaction buffer; and 5 µl of p32 labelled nucleotide α dATP (Pharmacia Biotech). The mixture was briefly spun to collect it in the bottom of tube, 2 µl Klenow fragment was added, and the sample was mixed and incubated at 37 °C for 10 min. After adding 45 µl of dH_2O, the mixture was spun through a

G50 Sephadex spin column. The eluted probe was boiled for 5 min, placed on ice for 5 min, and finally added to the hybridisation solution.

2.2.3 RNA extraction and Northern blot analysis

Total RNA extracts were made from different cell types using the TriZol reagent (Invitrogen, Cat # 15596-018,). Suspension cultures of each cell type (5-10 x 10^6 cells) were pelleted, 1 ml TriZol was added and the cells were homogenized by passing through a pipette several times. The samples were incubated at room temperature (RT) for 5 min and 0.2 ml chloroform was added followed by vigorous shaking for 15 seconds. After 3 min RT incubation, the sample was spun at 12,000 g for 15 min at 4°C. The aqueous upper phase containing the RNA was then transferred to fresh reaction tubes and precipitated by adding 500 µl isopropanol. Samples were incubated at RT for 10 min and then spun at a 12,000g for 10 min at 4 °C. The precipitated RNA was washed in 1 ml of 75% ethanol, vortexed briefly and re-spun at 7500g for 5 min at 4 °C. The RNA samples were air dried for 15 min.

Fresh total RNA extracts were separated on 1.2% agarose/formaldehyde (FA) gel. DNA grade agarose (Progen) (1.32 grams) was dissolved in 110 mls RNAase-free water and allowed to cool to 65 °C in a water bath, 1.98 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution were added, and the solution was mixed thoroughly and poured onto gel support. Prior to applying samples, the gel was equilibrated by running in 1x FA gel running buffer (see **2.1.7**) for at least 30 min. The RNA samples (5 µg) were dissolved in 5x RNA loading buffer, loaded onto the pre-equilibrated gel and electrophoresed at 5-7 V/cm in 1X FA gel running buffer 2 hr. The samples were then transferred to a N+

membrane (Amersham Pharmacia) by capillary action overnight using a 20x SSC solvent. The membrane filter was UV cross-linked twice at 1200 mega-joule using StrataGene UV cross linker for 60 seconds each, and then prehybridised in ULTRAhyb™ buffer (Ambion, Cat # 8670) for at least 30 min at 42 °C in a Robins Scientific Hybridisation Oven. Denatured DNA probe (see **2.2.2**) was added directly to prehybridisation buffer, together with 300 µl of 10 mg/ml salmon sperm that had been heat denatured separately at 85 °C for 5 min. Hybridisation was carried out at 42 °C in a hybridisation oven for 12-24 hr. The membrane was then washed in 2x SSC plus 0.1% SDS at 42 °C for 30 min, 0.2x SSC plus 0.1% SDS at 50 °C for 30 min, and finally 0.2x SSC plus 0.1%SDS at 60 °C for 30 min. The membrane was scanned by a Typhoon 9410 variable mode imager (Molecular Dynamics) and the ^{32}P-labelled bands were visualised and quantified.

2.2.4 Polymerase Chain Reaction (PCR)

For each reaction, 1 µl (5 U/µl) of polymerase Taq Gold (Roche, Part No. B08345) was added to a final volume of 100 µl containing a pair of primers (15~20 pmol each), 100 ng template DNA, 8 µl 25 mM $MgCl_2$, 8 µl 2.5 mM deoxynucleotide triphosphates (dNTPs), and 10 µl 10 X PCR buffer. PCR was performed for 30-35 cycles using the Perkin-Elmer GeneAmp PCR system 2400 (Perkin-Elmer Corporation). The programs for each PCR were varied slightly depending on the template or primer characteristics. PCR amplified DNA was examined by 1% agarose gel electrophoresis (100 volts) and used for further experiments.

2.2.5 Determination of the full-length Triad 3au cDNA sequence

Total RNA was extracted from A3.01 cells using TriZol reagent (section **2.2.3**), and the purified RNA and also total RNA from human placental tissue (provided by Clontech as a positive control) were pretreated with DNAase to exclude contaminating human chromosome DNA. These were then used for further RT PCR reactions. The cDNA was synthesised by using SMART™ RACE cDNA Amplification Kit (Clontech Cat # 634914) following the protocol of the manufacturer. Total A3.01 RNA (50 ng-1 μg in 4 μl) was mixed with 1μl 3'-CDS primer A in a 0.5 ml microcentrifuge tube and the mixture was incubated at 70°C for 2 min and cooled on ice for 2 min. Next, 2 μl 5x 1° strand-buffer, 1μl DTT (20 mM), 1 μl dNTP Mix (10 mM) and 1μl Powerscript Reverse Transcriptase were added to the mixture followed by a 1.5 hr incubation at 42 °C. A positive control was set up in parallel using the human placental RNA contained in same kit. Both RNAs were reverse transcribed in duplicate reactions, differing by the presence of either 1μl Powerscript Reverse Transcriptase or 1 μl sterile water. The reaction product was diluted 1:5 with Tricine-EDTA buffer contained in the kit, and heated for 7 min at 72°C. The product was then kept at –20°C for future use. These newly synthesised cDNAs were used as templates for further nested PCR experiments. Since data searches indicated that Triad 3au represented an inner section of the full length ZIN gene, two pairs of primers were synthesised based on reported ZIN nucleotide sequence information (Chen et al., 2002). In particular the inside forward primer ZnE (+) contained an *EcoR I* site at its 5' end and the reverse primer ZnB (-) contained a *BamH1* site at its 3' end. The PCR was performed using Taq gold as described in section **2.2.4.** The PCR product was then treated with restriction enzymes *EcoRI* and

BamHI and subcloned into p3Flag expression vector. This construct was confirmed by sequencing described in next section.

2.2.6 DNA constructions

The mammalian plasmid expressing HA-tagged Triad 3au was produced by PCR amplification of Triad 3au cDNA isolated from a human leukocyte cDNA library (Clontech) using the flanking sequence specific primer pair TrE (+) and TrX (-). The PCR product was digested by restriction enzymes *EcoRI* and *XhoI* and cloned downstream of the hemagglutinin (HA) epitope tag sequence and the cytomegalovirus (CMV) immediate early promoter of pCMV-HA (Clontech). Similarly NVBP cDNA (Lake et al., 2003) was PCR amplified by using primer pair NvE (+) and NvX (-), and the PCR product was digested by the same restriction enzymes, cloned into pCMV-HA and named pHA-NVBP.

Full-length ZIN cDNA prepared by PCR from cDNA synthesised from total RNA extracted from A3.01 cells (see section **2.2.5**) was subjected to restriction enzyme digestion with *EcoRI* and *BamHI* and cloned downstream of the Flag epitope sequence and CMV promoter in p3XFlag-CMV-10 (Sigma) to generate the expression plasmid encoding ZIN (pFlag-ZIN). The expression plasmid expressing HIV Vif (pMyc-Vif) was generated by cloning the full-length *vif* gene, amplified by PCR from pNL4.3 HIV-1 plasmid DNA using primer pair Vif Eb (+) and Vif S (-) and digested by *EcoRI* and *SalI*, into the protein expression plasmid pCMV-Myc (Clontech), downstream of the c-Myc epitope tag sequence. In addition, to express a GST-Vif fusion protein in *E.coli* under the control of the chemically inducible *tac* promoter, the Vif gene was amplified with primer pair VifB (+) and VifE (-), digested by *BamHI* and *EcoRI* and cloned into the plasmid pGEX 2T (Amersham

Biosciences) downstream of the glutathione S-transferase (GST) sequence (hereafter called pGST-Vif). All restriction enzymes for producing the above constructs were purchased from BioLabs. The cDNA sequences of all the above constructs were confirmed by sequencing using ABI Prism® BigDye Terminator Sequencing reaction kits (PE Applied Biosystems) and plasmid-specific forward and reverse primers. Homology search of nucleotide and deduced protein sequences was performed using the GenBank™ database.

2.2.7 Transient cell transfection

For DNA transfection of human 293T cells, cells were seeded in 100-mm-diameter dishes at a density of $2x10^6$ cells in 12 milliliters of DMEM cell culture medium, and maintained at 37 °C, 5% CO_2. On the following day, separate cultures were used for transfection. To examine the interaction between Vif and Triad 3au or NVBP in 293T cells, cells were singly transfected with 5 μg plasmid DNA, eg. pMyc-Vif, pHA-Triad 3au or pHA-NVBP using SuperFect® transfection reagent (Qiagen Cat # 301305) according to the manufacturer's recommendations. In cotransfection experiments, separate cultures were co-transfected with 5 μg each of pMyc-Vif and pHA-Triad 3au plasmid DNA, or 5 μg each of pMyc-Vif and or pHA-NVBP. To examine whether Vif interacted with ZIN in 293T cells, cotransfection was performed using the same procedure described above but replacing pHA-Triad 3au with pFlag-ZIN. As a negative control, 5 μg of each pMyc-Vif and pFlag plasmid were co-transfected. For GST pull-down experiments, Flag-ZIN protein was also produced in 293T cells, and 10 μg of pFlag ZIN was used for transfection. All transfected cell cultures were split and re-seeded into two 100 mm diameter dishes at 24 hr after transfection and incubated for a further 24 hr before harvesting.

2.2.8 Western Blotting

Polyacrylamide gels were set-up using the Bio-Rad gel preparation and electrophoresis apparatus. Gels consisted of a 12% resolving layer and a 5% stacking layer. The resolving gel was formulated to contain 9.9 mls dH_2O, 12 mls 30% acrylamide solution (1g Bis-acrylamide, 72.5 mls 40% acrylamide (Bio-Rad), and 27.5 mls dH_2O), 7.5 mls 1.5 M Tris pH 8.8 and 300 µl 10% SDS (5 grams of SDS in 50 mls dH_2O). The gel was polymerised by addition of 300 µl of 10% APS w/v and 30 µl N,N,N',N',-tetramethylethylenediamine (TEMED) (Bio-Rad). The stacking gel was formulated to contain 6.8 mls dH_2O, 1.7 mls 30% acrylamide solution, 1.2 mls of 1M Tris pH 6.8, and 100 µl of 10% SDS, and was polymerised by addition of 100 µl of 10% APS and 10 µl of TEMED.

After boiling for 5 min, 60 µg protein samples were loaded onto the gel and the gel was run at 160 volts for 3 hr. Proteins were then electroblotted onto Hybond^{TM}–C membrane (Amersham) at 75 mA for 1.5 hr (transfer buffer see **2.1.7**). The membranes containing transferred proteins were blocked in TBST containing 5% milk for 30~60 min before incubating with primary monoclonal antibodies or polyclonal antibodies. Under certain circumstances, blocking and incubation with primary antibodies were extended to overnight followed by 3 washes (15 min each) with TBST (see **2.1.7**). The secondary immunoreaction was performed using horseradish peroxidase-linked goat anti-mouse (PIERCE) or goat anti-rabbit IgG (PIERCE). After 3 washes (15 min each) with TBST, the blots were detected using SuperSignal West Dura Extended Duration substrate (PIERCE). X-ray film exposed to blots (Kodak X-Omat-K) was fixed and developed using an Ilfospeed 2240 X-ray film developer (Ilford).

2.2.9 Co-immunoprecipitation analyses

Transfected 293T cells from two separate 100-mm-diameter dishes were harvested and subsequently combined 48 hr after transfection. The cells were then lysed in 800 µl lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM EDTA plus protease inhibitor cocktail (Roche, 1 tablet for 10 mls lysis buffer). The lysates were sonicated for 3 separate 10 second pulses before centrifugation at 13,500 rpm at 4 °C for 10 min in a Biofuge 17 RS centrifuge (Hereaus) to remove cell debris. Clarified cell lysates were immunoprecipitated by adding rabbit polyclonal antiserum (1.8 µg anti HA) or a monoclonal antibody and then incubating for 12 hr at 4°C. Next, 100 µl of a 50% suspension of protein-A Sepharose® CL-4B beads (Pharmacia) was added and the reactions were incubated for a further 1 hr. The sepharose CL-4B beads containing immuno-protein complexes were pelleted, washed 3 times in lysis buffer, and then boiled in 100 µl denaturing SDS protein loading buffer. The eluted proteins were analysed by western blotting. Co-immunoprecipitated proteins present on the membrane were detected with mouse anti-Myc antibody or Vif specific rabbit antiserum followed by horseradish peroxidase-linked goat anti-mouse or goat anti-rabbit IgG (see section **2.2.8**). For Vif–ZIN co-immunoprecipitation analysis, the procedures were similar to Vif-Triad 3au co-immunoprecipitation with the exception of using different antibodies. Briefly, 2 µg Flag-specific monoclonal antibody was used for immunoprecipitating Flag tagged ZIN protein, and Vif specific rabbit polyclonal antibody was applied as a primary antibody in western blot analysis for examining co-immunoprecipitated Vif protein.

2.2.10 Immunofluorescent staining

To examine the localisation of Vif and Triad 3au/ ZIN in transfected cells, confocal microscopy was employed. The images were produced using the BioRad Radiance 2100 confocal microscope (Bio-Rad Microscience Ltd, UK) equipped with three lasers, Argon ion 488 nm (14mw); Green HeNe 543 nm (1.5 mw); Red Diode 637 nm (5 mw) outputs and an Olympus 1x 70 inverted microscope. The objective used was a 60x UPLAPO with NA=1.4 oil. The dual labelled cells were imaged with two separate channels (PMT tubes) in a sequential setting. The Alexa 488 was excited with Ar 488 nm laser line and the emission was viewed through a HQ515/30 nm narrow band barrier filter in PMT1. The Alexa 546 was excited with Green HeNe 543 nm laser line and the emission was viewed through a long pass barrier filter (570 LP) to allow only red light wavelengths automatically pass through PMT2. All signals from PMTs 1 and 2 were merged. The image data were stored on a CD for further analysis using a confocal Assistant software program for Microsoft® Windows™ (Todd Clark Brelje, USA). 293T cells were transfected with pMyc-Vif and pHA or pMyc-Vif and pFlag control plasmid, or pFlag-ZIN and pMyc control plasmid (also see results section), or co-transfected with pMyc-Vif and pHA-Triad 3au or pMyc-Vif and pFlag-ZIN using the SuperFect transfection reagent as described by the manufacturer. On the next day, the transfected cells were split and re-seeded on glass coverslips for 24 hr, before being fixed to the coverslips with 1% formaldehyde for 30 min at room temperature and by permeabilised in 0.05% IGEPAL (SIGMA) for 20 min at room temperature. Duplicate, but non-permeabilised, coverslips were used for detection of surface membrane protein staining only. Immunostaining was performed by sequential incubation with primary Vif-specific rabbit polyclonal antibody or mouse anti-Flag antibody or mouse anti-

HA antibody, followed by Alexa Fluor 546 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies. For nuclear staining, cells were stained with 5 μg/ml Hoechst 33342 (Molecular Probes) for 20 min at room temperature. The dually labelled cells on coverslips were mounted using prolong antifade media (Cat # P-7481, Molecular Probes) and sealed to slides using nail polish oil for confocal microscope examination. Confocal images of cells were obtained using a BioRad Radiance 2100 confocal microscope.

2.2.11 GST fusion protein pull-down assay

Plasmids pGST and pGST-Vif were transformed into *E.coli* BL21 cells using the $CaCl_2$ method. At an optical density (OD_{600}) of 0.8, the cells were induced with IPTG (0.2 mM final concentration) at 30°C for 3 hr. The cells were then collected by centrifugation at 6000 rpm in a JA-20 rotor (J2-21M/E centrifuge, BECKMAN) for 30 min at 4 °C. Cell pellets were lysed at 4°C for 30-60 min using lysis buffer containing 25 mM Tris/HCl pH7.4, 150 mM NaCl, 10% glycerol, 1 mM DTT, protease inhibitor cocktails (Complete Mini; Roche) and 0.05% Triton X-100. The lysates were then subjected to centrifugation (Biofuge 17 RS centrifuge) at 13,500 rpm, 4°C for 10 min to remove cell debris. Since the level of GST-Vif protein expressed in *E.coli* BL21 was much lower than the level of GST protein, the GST protein used as negative control was adjusted to the same level as GST-Vif protein. To quantitate the amount of protein, serial dilutions of GST protein together with equal volume of GST-Vif protein were resolved in SDS-PAGE gels and stained with coommassie-blue (also see section **4.2**). Thus, to perform pull-down assay, 800 μls of GST-Vif protein lysate and 800 μls of GST protein lysate (16 uls of original GST protein lysate was added to 784 uls of lysis buffer, 1:50 dilution) were incubated with

100 µls Glutathione-Sepharose CL-4B beads (Pharmacia Biotech) at 4°C for 3 hr and the beads containing the complexed GST-Vif fusion protein or the complexed GST protein were pelleted and extensively washed with lysis buffer.

Next, 293T cells were transfected with the pFlag-ZIN expression plasmid as in **2.2.7** and then lysed in the same lysis buffer as described in the above paragraph followed by centrifugation in a Biofuge 17 RS centrifuge at 13,500 rpm, 4°C for 10 min to remove cell debris. Fresh 50 µl (or 100 µl 50% slurry) glutathione-Sepharose CL-4B beads were added to the cell lysates, incubated at 4 °C for 1 hr and then removed by centrifugation and this step was repeated once using 50 µl of fresh beads complexed with GST protein. The aim of this procedure was to remove non-specific intermediate proteins in the lysates that might have been also pulled down with the beads. Equal quantities of glutathione Sepharose CL-4B beads (50 µl) complexed with GST-Vif fusion protein (~2 µg), or beads complexed with GST alone (~2 µg protein), were added to separate samples of pre-cleared pFlag-ZIN cell lysates. The mixtures were then incubated 2-3 hr at 4°C to allow opportunity for interaction between pFlag-ZIN in the cell lysates and proteins bound to the beads. The glutathione Sepharose CL-4B beads together with their bound proteins ('pull-downs') were then pelleted at 2,000 rpm in a microcentrifuge. The pellets were washed 3 times using the same lysis buffer, and the bound proteins were released by boiling and separated on SDS PAGE gels. As described in section **2.2.8**, proteins were then electroblotted onto Hybond^{TM}–C membrane (Amersham) at 75 mA for 1.5 hr (transfer buffer see **2.1.7**). The proteins were analysed by western blotting using anti-Flag antibody to detect ZIN, anti-GST antibody to detect GST protein or the GST-Vif fusion protein, and finally anti-Vif polyclonal antibody to detect Vif

(section **2.1.6**). These assays were performed sequentially by using the same filter with successive stripping of the previous antibody (incubated in 50 mM Tris pH 7.5, 2% SDS and 37 mM β-mercaptoethanol 50°C for 20 min followed by 3 times washes with TBST).

2.2.12 Production of HIV NL4.3 stocks

HIV viral stocks were produced in 293T cells. Briefly, 293T cells were co-transfected with plasmid pNL4.3 (5 μg) and plasmid pFlag-ZIN (5 μg) or plasmid pNL4.3 (5 μg) and control plasmid pFlag (5 μg), or Δ*vif* pNL4.3 (5 μg) and pFlag-ZIN (5 μg), or Δ*vif* pNL4.3 (5 μg) and pFlag (5 μg) in 100-mm-diameter plates, and the culture supernatants were collected 48 hr after transfection. The supernatants were filtered using a 0.2 μm filter, 10-fold diluted in triplicate. HIV p24 levels in the virus culture medium were measured from serial dilutions (inactivated by treating 285 μl of each dilution with 15 μl 10% Triton X-100 at 4°C overnight) by ELISA analysis using HIV-1 p24 analysis kit (PerkinElmer™, Cat # NEK050B). These viral stocks were stored at –70 °C for further experiments.

2.2.13 Single-cycle viral infectivity assay

Single –cycle viral infectivity assays were carried out in HeLa-CD4-LTR-β-gal cells (Kimpton & Emerman, 1992), grown as described in section **2.1.1**. Individual wells of a 48-well plate were pre-seeded with $1.5x10^4$ HeLa-CD4-LTR-β-gal cells in 300 μls medium A (see section **2.1.1**) on the day before infection. On the next day, medium A was removed and cells (approximately 20% confluent) were washed twice in PBS. The cells were infected with 100 μls/well of neat and virion dilutions from

10^{-1} to10^{-3} (3 wells per dilution). To make these dilutions, 70 (or 35) µls of 400 ng/ml p24 virus stock (neat) was diluted in 630 (or 315) µls medium B (DMEM 50 mls containing no serum or antibiotics but 1 mg DEAE Dextran). Cells treated with medium B containing no virus were used as negative control. The cells were incubated at 37°C for 2-4 hr at normal cell culture condition followed by three washes in PBS. These cells were then incubated in fresh culture medium A for a further 30 hr, then washed in PBS and fixed in 1% formaldehyde in PBS for 10 min. The cells were again washed 3x in PBS and then incubated in X-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM $MgCl_2$ and 1 mg/ml X-gal) for exactly 50 min. The staining solution was removed and cells were washed twice in PBS. Finally blue colonies were counted under a microscope, and wells corresponding to the virus dilution in which at least 80-100 blue colonies per well were present were used for quantitation of infectivity.

2.2.14 Viral titres ($TCID_{50}$)

For $TCID_{50}$ titration, virus dilutions were generated from the same stocks using the same method described in section **2.2.13.** except that viral stocks were diluted from 10^{-1} to 10^{-5}. Neat and each dilution were incubated with $1.5x10^5$ HuT-78 cells at 37°C for 2 hr. The cells were washed twice in fresh medium and resuspended in 1.2 ml medium, and then 200 µl aliquots were plated out into 6 individual wells of a 48-well plate. Six days post infection (pi) the supernatants were inactivated using the same method described in section **2.2.12.** These mixtures were then assayed for p24 level by ELISA. Each well was scored positive or negative based on viral p24 amounts. The virus titre, expressed as $TCID_{50}$/ml, was then calculated from dilution

of the virus stock at which 50% of wells were infected using the protocol available in HIV laboratory of IMVS (Procedure No. RHIV 01-05 V1).

2.2.15 Extent of reverse transcription in newly infected target cells

To measure the levels of reverse transcription in newly infected HuT-78 cells, in culture supernatants from 293T cells co-transfected with either pNL4.3 or pNL4.3 (*ΔVif*) together with either pFlag or pFlag-ZIN were used as virus inocula. Equivalent amounts of each inoculum (measured as 10 ng of HIV p24) were separately added to HuT-78 cells (1.5 x 10^5), and virus adsorbed by centrifugal enhancement for 1 hr at 37°C (Vandegraaff et al., 2001). Cells were then washed twice in fresh medium and divided into duplicate wells. The wells were harvested 16 hr and 24 hr pi and unintegrated low-molecular-weight DNA was extracted by the Hirt method as follows. Infected cells were spun at 5,000 rpm in a microcentrifuge for 2-3 min, the medium was removed, and the pellets were flicked and subsequently resuspended in the remaining media. PBS 800 µl was then added to resuspend cells followed by a low spin as above. Supernatants were discarded and the pellets were resuspended in 160 µl Hirt solution I (section **2.1.7**). Next, 20 µl of proteinase K (10 mg/ml) was added, followed by 200 µl of Hirt II solution (section **2.1.7**). The mixtures were incubated at 37°C for 30 min, 100 µl of 5 M NaCl was added, and the mixture was incubated at 4°C for more than 3 hr. All samples were spun at 17,000g at 4°C for 45 min, the supernatants were transferred to new tube, and DNA was extracted using the conventional phenol/choroform/IAA (25:24:1) method. To minimise sodium dodecyl sulfate contamination of the DNA preparations, all ethanol precipitations were performed at room temperature. DNA preparations were resuspended in water (Vandegraaff et al., 2001).

HIV DNA levels present in the Hirt supernatant were quantified against pre-titrated HIV copy number standards (Vandegraaff et al., 2001) by real-time PCR using primer pair SS (+) and SS (-) specific for strong-stop DNA (U5-U5), or primer pair NL4 (+) and NL4 (-) specific for the U5-Gag region reverse transcription product. Each real-time PCR reaction contained 5 µl of Syber Green (Molecular Probes), 0.75µl of primer pairs (10 pmol/µl), 1.5µl of H_2O and 2µl Hirt DNA. The PCR reaction was performed using Rotor Gene Real Time machine (Corbett Research). Results were analysed using Rotor-Gene Analysis Software V6.0.

2.2.16 Site-directed mutagenesis of HIV-1 Vif

Alexander *et al.* isolated a natural HIV-1 strain (AY064706) that contained two extra amino acids (DS) after the position 61 of Vif, leading to poor replication of the virus in human PBMC cells (Alexander et al., 2002). A Vif mutant termed "DS mutant" was created in this study using the overlap insertion PCR technique. Four primers were designed based on Vif sequence information. Primer Vif DS (A) contained the *EcoRI* enzyme digestion site, and primer Vif DS (B) had 6 extra nucleotides corresponding to the two extra amino acids. They were used for amplification of the upstream region of Vif DS DNA (named fragment AB). The downstream region of Vif DS (named fragment CD) was amplified using primer Vif DS(C) containing 6 nucleotides complementary to the extra nucleotides contained in Vif DS (B), and primer Vif DS (D) specific to the 3' terminus of Vif sequence containing an introduced *Sal*I enzyme digestion site (see Chapter 4). Fragments AB and CD obtained from the first round were then used in the second round PCR as template DNA, in subsequent cycles, flanking primers Vif DS (A) and Vif DS (D) primed

DNA synthesis (refer to Figure 5.1). Two rounds of PCR were performed using conventional DNA amplification technique with Taq gold (section **2.2.4**).

Chapter 3

Interaction between HIV-Vif and Triad 3au

3.1 Introduction

Replication of human immunodeficiency virus type 1 (HIV-1) in all primary cells and in some but not in all immortalized T cell lines, depends on the activity of the viral infectivity factor (Vif). Cell fusion experiments have shown that Vif is required to counteract a cellular inhibitor present in non-permissive cells (Madani & Kabat, 1998, Simon et al., 1998a). As described above, a number of cellular factors have been proposed to be related to this function of Vif. The most convincing factor of these is APOBEC3G, which was recently identified as an inhibitor that blocks infectivity of Vif-defective HIV-1 variants (Sheehy et al., 2002). More recent studies have described possible mechanisms whereby Vif can overcome APOBEC3G and suggested some unidentified cellular clones may involve in Vif function, including later identified Skp1-cullin-F-box (SCF)-like complex (Yu et al., 2003). The exact mechanism whereby Vif overcomes APOBEC3G has not been fully elucidated, and the full biological relevance of other Vif interacting factors is not clear. Work in this thesis using the Yeast-2-hybrid system to identify Vif-interacting cellular factors began in 2001 before APOBEC3G was identified.

The Yeast 2-hybrid system has been used successfully for identification of cellular proteins interacting with a variety of HIV-1 proteins (Howe et al., 1998, Mahalingam et al., 1998). Notably the interaction between cyclophilin A and Gag was identified in this manner and subsequently found to be important for viral infectivity (Franke et

al., 1994, Luban, 1996). In previous preliminary studies, our laboratory has employed this system to identify cellular proteins which interact with HIV-1 Vif. Sixteen clones were isolated from a high stringency yeast-2-hybrid screen of a human leucocyte cDNA library with Vif derived from the T-cell tropic HIV-1 strain NL4.3. Of these, eight clones confirmed as binding Vif were fully sequenced and identified via GenBank™ homology searches (Lake et al., 2003). This thesis focuses on two of these clones: a novel Vif binding protein termed NVBP, and Triad 3au related to a previously identified protein Triad 3 (Lake et al., 2003, van der Reijden et al., 1999).

3.1.1 NVBP

Lake *et al.* (Lake et al., 2003) sequenced the entire clone NVBP to ascertain intron/exon boundaries, and preliminary protein modelling showed that this protein has distant yet significant homology to an uncharacterised *Drosophila* ring finger protein. To confirm whether NVBP is expressed in cultured cells, Lake *et al* extracted the total RNA from primary cells and cell lines and subjected these RNAs to Northern analysis. A mRNA of approximately 2.1kb was detected indicating that NVBP represents a genuine cellular transcript. Of major interest was the observation that NVBP mRNA was found in non-permissive but not permissive cells (Figure 3.1) (Lake et al., 2003). Thus, expression of NVBP was consistent with that of potential Vif-related cellular inhibitor, and it was selected for further study.

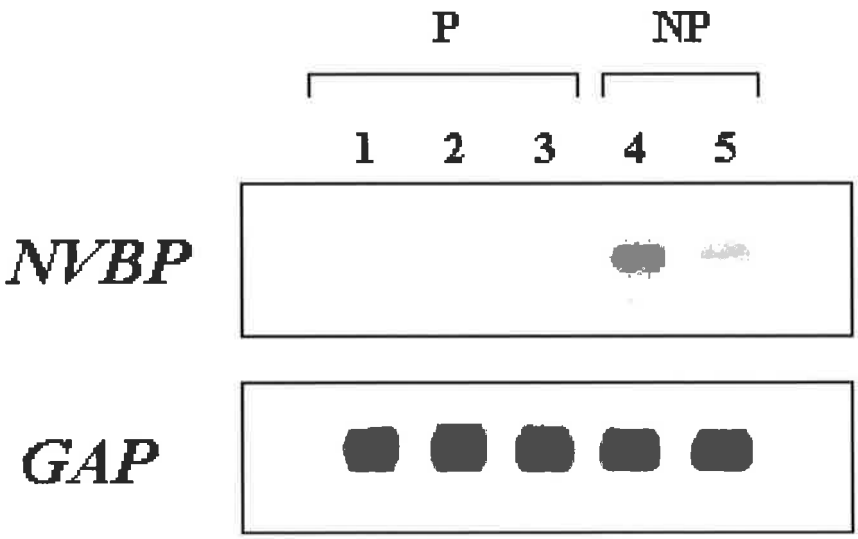
3.1.2 Triad 3au

Triad 3au is a partial clone exhibiting homology to a 218 amino acid region of a hypothetical protein Triad 3 reported by a Dutch group (van der Reijden et al., 1999).

Figure 3.1 Northern analysis of expression of NVBP

The expression of NVBP in total RNA (10 ug) derived from permissive cells (P). 1: HeLa, 2: CEM-ss, 3: COS7 and non-permissive cells (NP) 4: MDM, 5: HuT-78, GAPDH mRNA was used as a control for equivalent loading in each sample.

Adapted from Lake et al.(2003)


P
NP
1
2
3
4
5
NVBP
GAP

Initially, Triad 1 in acute leukemia cells was identified as a nuclear RING finger protein possessing a unique ring-finger motif by van der Reijden *et al.* Following the initial identification, van der Reijden *et al* also identified 23 other protein (van der Reijden et al., 1999). The TRIAD family of proteins possess a novel ring finger structure or DRIL or "double-ring finger linked" motif characterized by 6 cysteine and 6 histidine residues flanked by 2 RING domains to form a triad. Triad 1 was shown to be upregulated during retinoic-induced granulocytic differentiation of acute promyelocytic leukemia cells (van der Reijden et al., 1999). Further, based on the presence of ring finger structures, all Triad containing proteins were hypothesised to mediate protein-protein interactions (van der Reijden et al., 1999). Following the initial report of Triad 3 sequence (AF228527), other homologous sequences became available in the database including, from Japan (AK024787) and the USA (BC000787). Both the Japanese and USA sequences contained a poly A sequence at their 3' ends which Triad 3 did not. All Triad 3 related homologue sequences including our Triad 3au sequence encoded the characteristic ring finger structures but their translation initiation sites were not known.

Our previous studies using the yeast two-hybrid system showed that Triad 3au interacted with Vif but not with the mutant HIV Vif protein, Vif^{M26} carrying substitutions in residues 105-107 in the Vif protein (Figure 3.2) (Lake et al., 2003). Vif^{M26} mutant virus has been shown to exhibit a marked reduction in infectivity on H9 cells (Simon et al., 1999b). Therefore, further characterizing the interactions between Triad 3au and Vif and investigating Triad 3au protein in HIV-1 susceptible cells was also a focus of this study.

Figure 3.2. Interaction of Triad 3au with Vif mutants

Mutants and their relative infectivity were firstly described by Simon et al (1999). The characteristics of the interactions between these mutants with Triad 3au were assayed using the Matchmaker yeast 2-hybrid system 3 (Clontech) by Lake J.

Adapted from Lake et al.(2003)

Name	Position	Mutation	Relative Infectivity	Interaction with Triad 3au
WT *vif*	NA	deletion	*high*	yes
Δ*vif*	28-192	deletion	-	no
M21	38-40	WFY-AAA	-	yes
M26	105-107	QLI-AAA	-	no
M29	144-146	SLQ-AAA	-	yes
M14	161-163	PPL-AAA	-	yes
Δ*vif* 172	172-192	deletion	*low*	yes
Δ*vif186*	186-192	deletion	*high*	yes

Not all interactions in the yeast two-hybrid system represent biologically significant interactions in mammalian cells. Since clones Triad 3au and NVBP had been shown to interact with Vif in yeast cells, the first aim which forms part of Chapter 3 was to re-examine our original finding of an interaction between these isolated clones and Vif using the yeast two-hybrid system. If confirmed, these interactions would then be studied further.

Within different cell systems it is conceivable that differences in posttranslational modifications or other cooperating proteins may significantly affect the interactive potential of two proteins. Therefore it was also necessary to examine whether Vif could interact with Triad 3au and NVPB in human cells as had been previously identified in yeast cells.

Finally, the subcellular localization of Vif was examined to elucidate cellular pathways which might be important for Vif function. Early studies suggested that Vif was predominantly associated with the Golgi complex (Guy et al., 1991). However, another immunofluorescence study, using COS cells transfcetd with Vif and HIV-1 infected Jurkat or CEM cells, demonstrated Vif localisation in the cell cytoplasm, cytosol and cytoplasmic side of the plasma membrane (Goncalves et al., 1994). Vif has also been reported to localize in the cytoskeleton (Karczewski & Strebel, 1996) and nucleus (Chatterji et al., 2000, Goncalves et al., 1995, Simon et al., 1997). The definitive cell localisation of Triad 3 has not been elucidated although protein structure analysis predicts that a large proportion of TRIAD proteins are nuclear (van der Reijden et al., 1999). Furthermore, if Vif interacts with Triad 3au in human cells, the intracellular distribution of Vif might be affected by this interaction. Conversely, the distribution of Triad 3au might be affected by the presence of Vif in infected

cells. These effects might mediate Vif function and affect HIV-1 infectivity in HIV-1 infection.

3.2 Interaction in yeast between HIV-1 Vif and Triad 3au or NVBP

The Matchmaker Two-Hybrid System 3 (Clontech) overcomes previous difficulties encountered with yeast 2-hybrid screens by using an extended reporter gene system to minimise detection of false positives. In MATCHMAKER System 3, a 'bait' gene (in this case Vif) is expressed as a fusion protein linked to the GAL4 DNA-binding domain (DNA-BD), while genes for the possible interacting proteins (as either the isolated gene or cDNA) are expressed by the 'prey' vector as fusion proteins linked to the GAL4 activation domain (AD). When the 'bait' (Vif) and candidate 'prey' proteins (eg Triad 3au or NVBP) interact, the DNA-BD and AD are brought into close proximity resulting in transcriptional activation of several reporter genes *HIS3/ADE2/LacZ* or *MEL1* (Figure 3.3). As described in section **2.1.4**, the 'bait' vector pGBKT7 contains the *TRP* gene, while the 'prey' vector pACT2 for expression of Triad 3au and NVBP contained the *LEU* gene. Thus, when both plasmids are successfully co-transformed into yeast AH109 cells and the expressed 'bait' and 'prey 'proteins interact, positive transformant yeast will be detected by high stringency screening on growth medium containing SD/-Ade/-His/-Leu/-Trp/X-α-Gal as blue colonies (Figure 3.4).

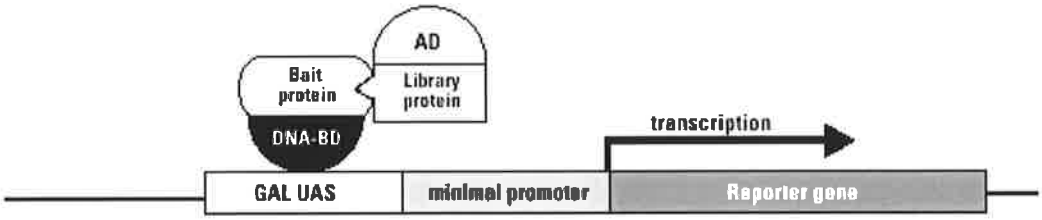
In this experiment, constructs pGKBT7-Vif and pACT2-Triad 3au or pACT2-NVBP were co-transformed into yeast AH109 cells. As negative controls, pGKBT7-Vif was also co-transformed with pACT2 vector, and pGKBT7 was co-transformed with

Figure 3.3 Overview of the Yeast 2 Hybrid System

Upper panel indicates that if the bait protein interacts with a library protein and brings the GAL4 DNA-binding domain (DNA-BD) and GAL4 activation domain (AD) into proximity, activation of transcription of the reporter genes will occur.

Lower panel shows the reporter genes contained in the construct AH109

Adapted from user manual PT3247-1 of Clontech http://www.bdbiosciences.com



AH109 Constructs

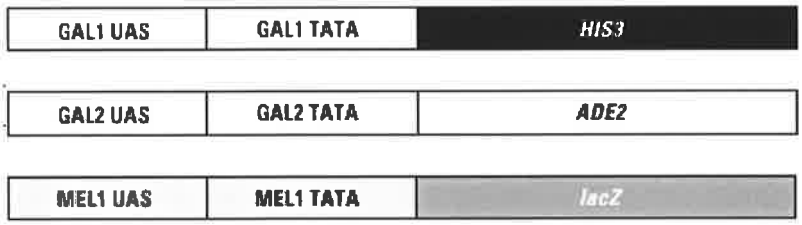
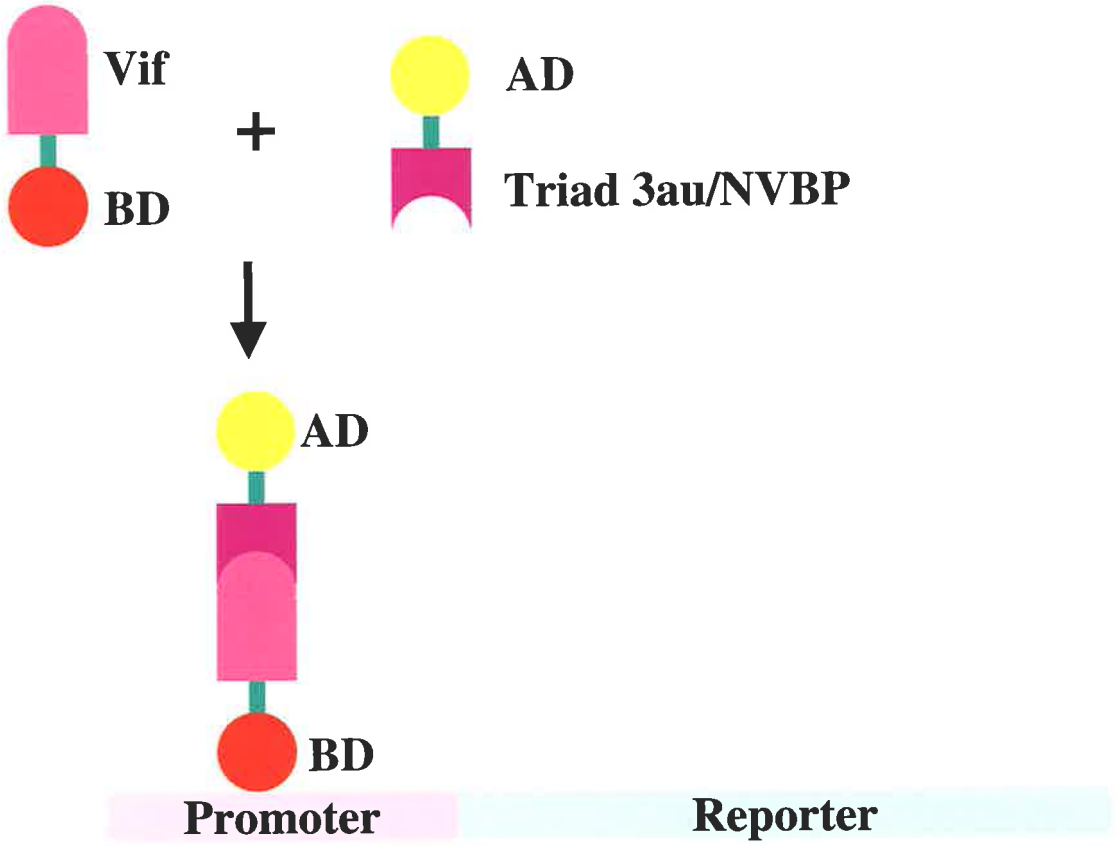


Figure 3.4 Principle of the Yeast 2 Hybrid System screen

The yeast two-hybrid technique uses two protein domains that have specific functions: a DNA-binding domain (BD), that is capable of binding to DNA, and an activation domain (AD), that is capable of activating transcriptions of the DNA. Both of these domains are required for transcription, whereby DNA is copied in the form of mRNA, which is later translated into protein. A bait gene (*vif*) is expressed as a fusion protein (pink) linked to GAL4 DNA-binding domain (DNA-BD, red), "prey" gene (Triad 3au or NVBP) is expressed as a linked protein (purple) to GAL4 activation domain (AD, yellow). If protein Vif and protein Triad 3au or NVBP (pink & purple) interact, then their BD and AD will combine to form a functional transcriptional activator. The transcriptional activator will then proceed to transcribe the reporter genes (*His, Ade, LacZ*), which are genes whose protein products can be easily detected.


Vif
BD
+
AD
Triad 3au/NVBP
AD
BD
Promoter
Reporter

either pACT2-Triad 3au or pACT2-NVBP. Next, yeast colonies were selected initially on SD/-Leu/-Trp agar media, and these were then streaked onto agar media containing SD/-Leu/-Trp/-Ade/-His/X-α-Gal. All transformants expressing both pGKBT7-Vif and pACT2-Triad 3au or pACT2-NVBP, or appropriate control plasmid combinations, were positively selected on SD/-Leu/-Trp agar media (Table 3.1). In contrast only transformants expressing fusion proteins capable of interacting would grow and appear blue on the selective media SD/-Leu/-Trp/-Ade/-His/X-α-Gal on account of their promoter induced expression of histidine (*HIS 3*), adenine (*ADE 2*) and β-galactosidase (X-α-Gal).

Table 3.1 Result of testing Vif –Triad 3au/NVBP interaction in yeast AH109

	SD/-Leu/-Trp	SD/-Leu/-Trp/-Ade/-His/ X-α-Gal
pGBKT7-Vif+pACT2 Triad 3au	yes	yes
pGBKT7-Vif+pACT2 NVBP	yes	yes
pGBKT7-Vif+pACT2 vector	yes	no
pGBKT7 vector+pACT2-Triad 3au	yes	no
pGBKT7 vector+pACT2 NVBP	yes	no
pGBKT7 vector+pACT2 vector	yes	no

* Yes: grow No: did not grow

As shown in table 3.1, transformants expressing either Vif and Triad 3au or Vif and NVPB, but not control vectors, grew on SD/-Ade/-His/-Leu/-Trp/ X-α-Gal selection medium and appeared blue colonies, indicating that both Vif and Triad 3au (or NVPB) proteins interacted in the yeast 2-hybrid system, confirming the earlier result of Lake *et al*, 2002. The sequences of the cDNA clones Triad 3au and NVBP were then determined using an ABI Prism® BigDye Terminator Sequencing reaction kit (PE Applied Biosystems). Both strands of each cDNA were sequenced and searched

for protein homologues in the GenBank™ database (see section **2.2.6**). The 654-nucleotide Triad 3au cDNA sequence was shorter than the full Triad 3 sequence but was identical to an internal segment, containing the ring finger domains of Triad 3. Figure 3.5 shows the sequence alignment of Triad 3au and Triad 3 reported by Van der Reijden *et al*, 1999.

The NVBP cDNA was determined to be 835 bps in length, and was located to human chromosome 15 (98% nucleotide sequence homology) in the GenBank™ sequence database (Figure 3.6) but did not correspond to any known protein.

3.3 Interaction between Triad 3au and HIV Vif in mammalian cells

To determine whether an interaction between Vif and either Triad 3au or NVPB occurred in mammalian cells, co-immunoprecipitation experiments were performed. Figure 3.7 shows the mechanism of this experiment. In summary, pairs of proteins were co-expressed in mammalian cells, the cells were lysed, and one of the protein species under study was immunoprecipitated using specific antibody and protein A sepharose / CL-4B beads. Every co-immunoprecipitated protein was then detected by western blot using a panel of antibodies.

The detailed procedure was as follows. The Vif gene was amplified from HIV-1 NL4.3 DNA by PCR (Figure 3.8A) and subcloned into pCMV-Myc with subsequent confirmation by restriction digest analysis using *EcoRI* and *Sal I* (Figure 3.8B). Similarly, NVBP (420 bps) and Triad 3au (654 bps) sequences were amplified by PCR from plasmid pACT2-NVBP and pACT2-Triad 3au (Figure 3.9) and subcloned

Figure 3.5 Amino acid sequence comparison of Triad 3au and Triad 3

AF228527 (GenBank™) indicates the Triad 3 amino acid sequence isolated by the Dutch group (upper) and Triad 3au shows the amino acid sequence of our isolate (lower). Triad 3au amino acid residues that are identical to the Triad 3 sequence are marked with dots.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
AF228527        DGQLIECRCC YGEFPFEELT QCADAHLFCK ECLIRYAQEA VFGSGKLELS
Triad 3au       ---------- -......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
AF228527        CMEGSCTCSF PTSELEKVLP QTILYKYYER KAEEEVAAAY ADELVRCPSC
Triad 3au       .......... .......... .......... .........P. ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
AF228527        SFPALLDSDV KRFSCPNPHC RKETCRKCQG LWKEHNGLTC EELAEKDDIK
Triad 3au       .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
AF228527        YRTSIEEKMT AARIRKCHKC GTGLIKSEGC NRMSCRCGAQ MCYLCRVSIN
Triad 3au       .......... .......... .......... .......... ..........

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
AF228527        GYDHFCQHPR SPGAPCQECS RCSLWTDPTE DDEKLIEEIQ KEAEEEQKRK
Triad 3au       .......... .......... ..........- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        260        270        280        290        300
AF228527        NGENTFKRIG PPLEKPVEKV QRVEALPRPV PQNLPQPQMP PYAFAHPPFP
Triad 3au       ---------- ---------- ---------- ---------- ----------

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        310        320        330        340        350
AF228527        LPPVRPVFNN FPLNMGPIPA PYVPPLPNVR VNYDFGPIHM PLEHNLPMHF
Triad 3au       ---------- ---------- ---------- ---------- ----------

                ....|...

AF228527        GPQPRHRF
Triad 3au       --------
```

Figure 3.6 NVBP homological sequence analysis

The upper lane indicates the NVBP clone nucleotide sequence. There is a stop codon located at position 420. NVBP is homologous to the region 122628-123463 of human Chromosome 15.

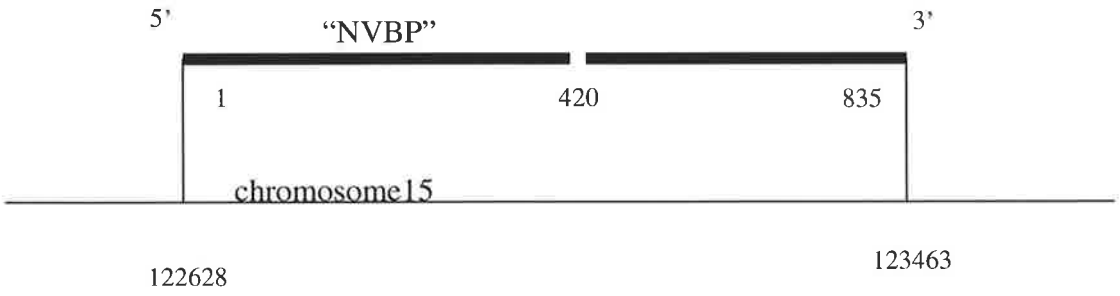

5'
"NVBP"
3'
1
420
835
chromosome15
122628
123463

Figure 3.7 Principle of co-immunoprecipitation (Co-ip)

Vif protein is tagged with Myc and Triad 3au protein is tagged with HA. If Vif interacts with Triad 3au, they will form a complex, which can be bound by HA antibody. When protein A beads are applied to this HA antibody/HA-Triad 3au/Myc-Vif complex and collected by centrifugation, Myc tagged bait protein (Vif) will be present in the immunoprecipitate complex bound to protein A beads via HA-Triad 3au.

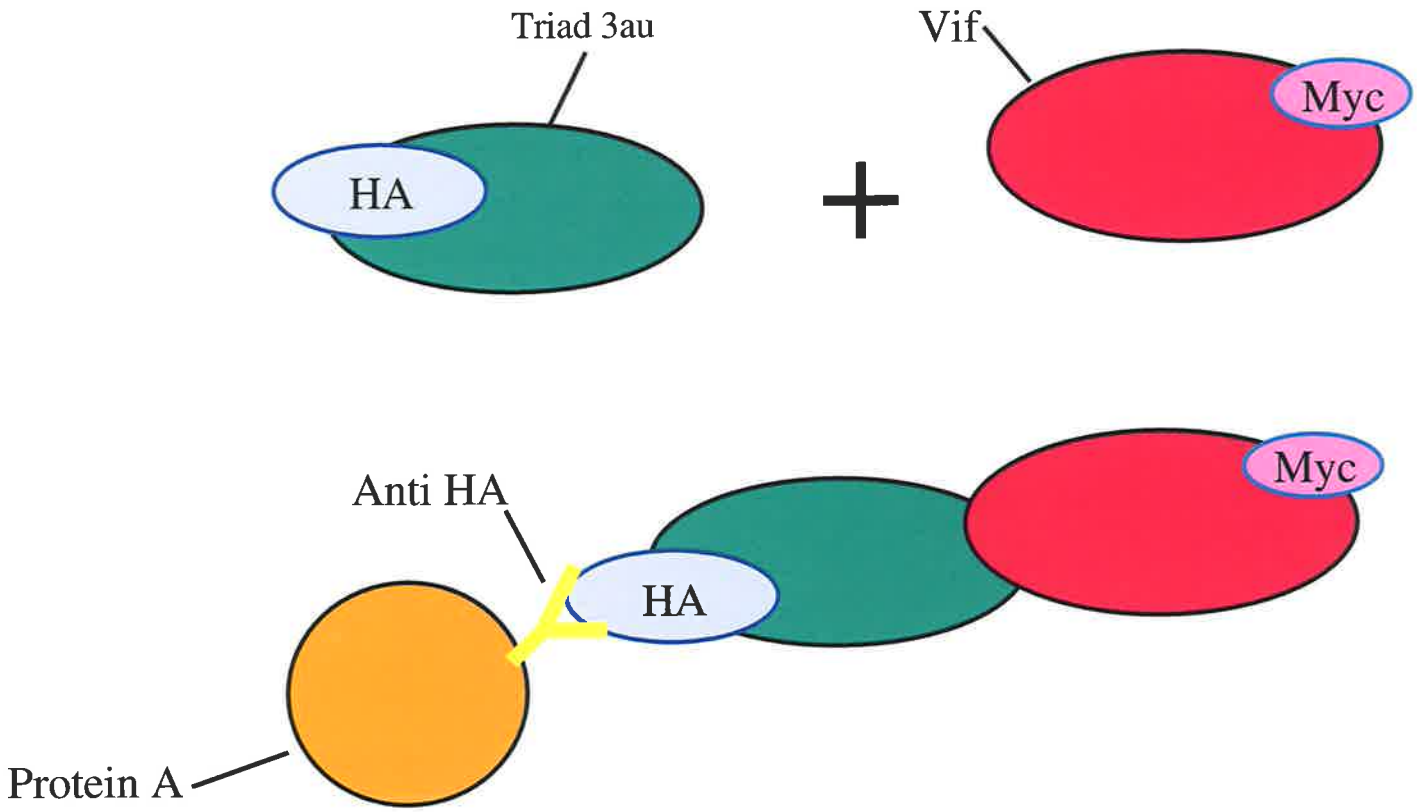

Triad 3au
HA
Vif
Myc
Anti HA
HA
Myc
Protein A

Figure 3.8 Vif detection using 1% agarose followed by ethidium bromide staining

A. Vif amplified from HIV-1 NL4.3 by PCR

B. Vif insert cut out from the construct pCMV-Myc-vif by enzymes *EcoR* I and *SaI* I double digestion

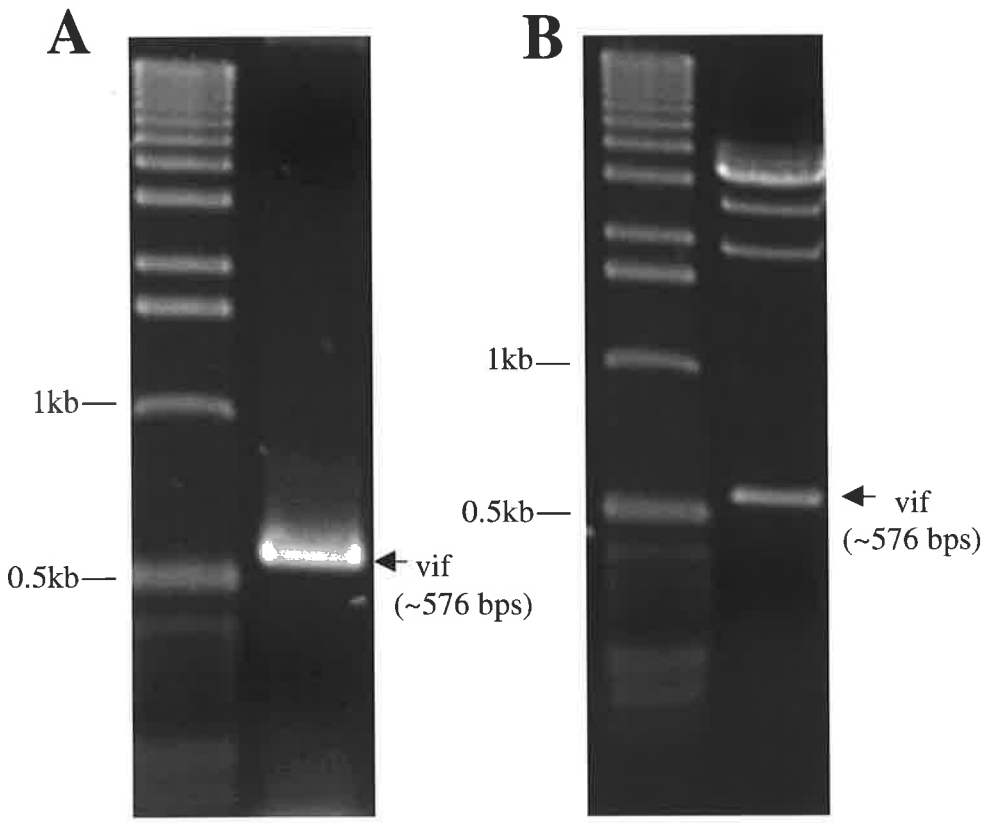
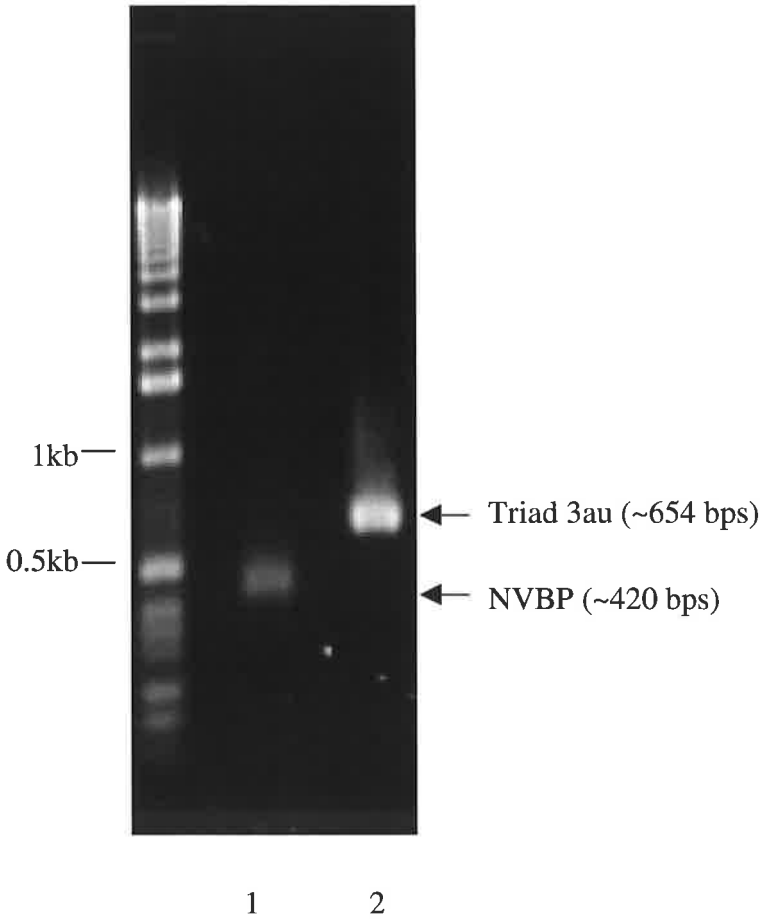

A
1kb
0.5kb
vif
(~576 bps)
B
1kb
0.5kb
vif
(~576 bps)

Figure 3.9 NVBP and Triad 3au (in frame) PCR products

Lane 1: NVBP

Lane 2: Triad 3au


1kb
0.5kb
Triad 3au (~654 bps)
NVBP (~420 bps)
1
2

into pCMV-HA, with subsequent confirmation by restriction digest analysis using *EcoRI* and *XhoI*.

293T cells were co-transfected with either pMyc-Vif and pHA-Triad 3au or pMyc-Vif and pHA-NVBP. Individual plasmids pMyc-Vif, pHA-Triad 3au or pHA-NVBP were also singly transfected to control for non-specific protein-protein interactions. Transfected cells exhibited abundant expression of HA-tagged Triad 3au, HA-tagged NVBP and Myc-tagged Vif by western blot using rabbit anti-HA antibody (Figure 3.10) or mouse anti-Myc antibody (Figure 3.11 right half). Proteins expressed in lysates from transfected cells were then immunoprecipitated with HA specific antibodies and immuno-complexed proteins pelleted by protein A sepharose / CL4B beads and analysed by SDS-PAGE and western blotting with anti-Myc antibody. Vif was readily detected in immunoprecipitates from lysates containing HA-Triad 3au (Figure 3.11; lane 5) but in comparison was not significantly detectable in immunoprecipitates from lysates containing HA-NVBP (Figure 3.11; lane 6). In contrast, when Vif was transfected alone, Vif was not detected in the HA specific immunoprecipitate (Figure 3.11; lane 2). To confirm the reliability of this co-immunoprecipitation result the experimental procedure was modified to comprise a murine monoclonal anti-HA specific antibody for immunoprecipitation and rabbit anti-Vif polyclonal antibody for western blot analysis. Vif protein was again readily detected by western blotting using the rabbit anti-Vif antibody in the Triad 3au containing immunoprecipitate but not in the NVBP containing immunoprecipitate (results not shown).

Figure 3.10 Triad 3au and NVBP expression analysis

Lysates to be for co-immunoprecipitation experiments were pre-screened for Triad 3au or NVBP expression using HA specific rabbit polyclonal antiserum.

1) untransfected cells
2) pMyc-Vif
3) pHA-Triad 3au,
4) pHA-NVBP,
5) pMyc-Vif + pHA-Triad 3au,
6) pMyc-Vif + pHA-NVBP

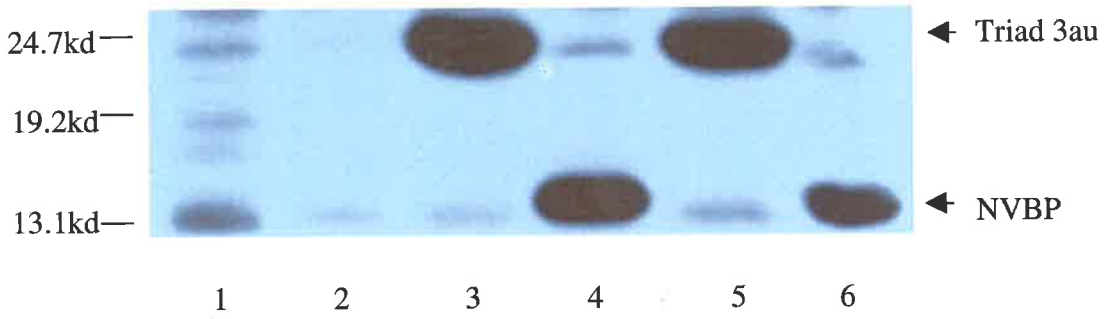
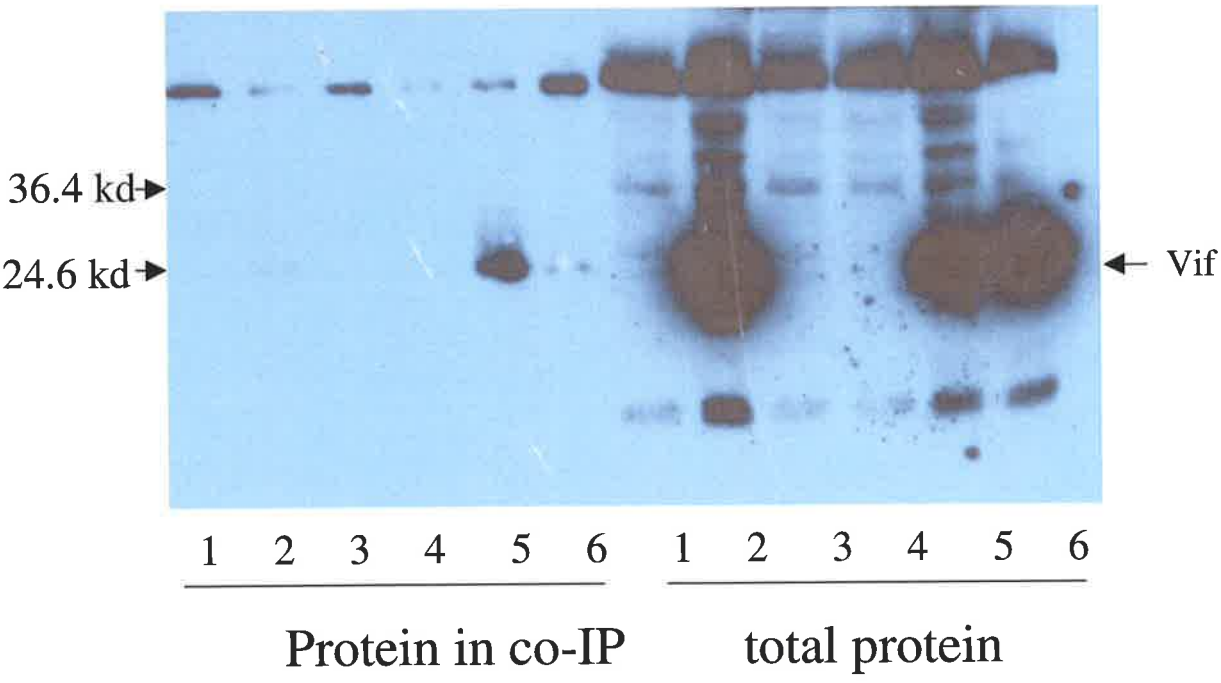

24.7kd
19.2kd
13.1kd
Triad 3au
NVBP
1
2
3
4
5
6

Figure 3.11 Co-immunoprecipitation of Vif and Triad 3au

Western blot analysis using monoclonal anti-Myc antibody. Right half of the figure indicates the total lysates of transfected 293T cells and left half of the figure shows the proteins coimmunoprecipitated by HA specific rabbit polyclonal antiserum.

1) untransfected cells
2) pMyc-Vif
3) pHA-Triad 3au
4) pHA-NVBP
5) pMyc-Vif + pHA-Triad 3au
6) pMyc-Vif + pHA-NVBP


36.4 kd
24.6 kd
Vif
1 2 3 4 5 6 1 2 3 4 5 6
Protein in co-IP
total protein

These results indicated that an interaction was occurring in mammalian cells between Vif and Triad 3au, but not between Vif and NVPB despite Vif/NVBP interaction demonstrated earlier using the yeast two-hybrid assay. As previously described, Triad 3au contains 4 ring finger sequence domains hypothesised to mediate protein-protein interactions. These results demonstrate that Triad 3au interacts with HIV-1 Vif in both yeast and mammalian cell types. It is not clear, however, which domain(s) of Triad 3au is required for this interaction, and more specific mapping experiments will be needed in order to fully characterise the interaction.

Unlike Triad 3au, NVBP did not interact significantly with Vif in mammalian cells. Differences in the cell milieu of yeast and mammalian cells may explain the discrepancy between the two expression systems. For example, yeast proteins might be involved in the interaction between Vif and NVPB in yeast, or alternatively posttranslational modification of NVBP in yeast might change the binding characteristics of NVBP protein favouring an interaction with Vif. This demonstrated that Vif could be co-immunoprecipitated by anti-HA polyclonal antibody in the presence of HA-Triad 3au but not HA-NVBP indicated that the interaction involved the Triad 3au moiety and not the HA epitope. pHA-NVBP might be suitable as a good negative control in this experiment. Since the interaction between Vif and NVBP could not be confirmed in mammalian cells, only the interaction between Vif and Triad 3au was pursued in remaining chapters of this study.

3.4 Effect of Triad 3au on the cellular localisation of Vif

Confocal laser-scanning microscope was employed to analyse the localisation of Vif and Triad 3au in cotransfected 293T cells. In this experiment, 293T cells co-

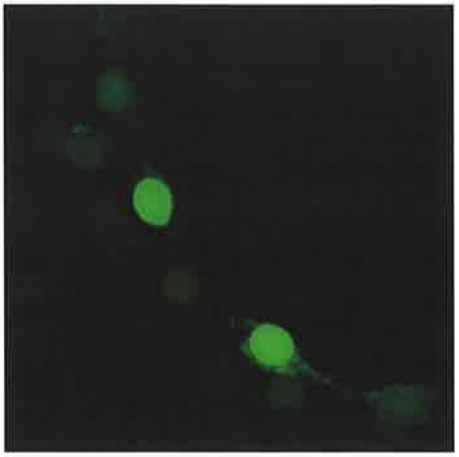
transfected with pMyc-Vif and pHA-Triad 3au were grown under normal growth conditions on a 100-mm-diameter dish. On the next day, the cells were split and plated on a fresh 100-mm-diameter dish containing sterile coverslips (section **2.2.10**). The cells were fixed 24 hr after splitting, incubated with primary antibodies (anti-HA monoclonal antibody or anti-Vif rabbit polyclonal antibody) followed by secondary antibodies (Alexa Fluor 546 goat anti-mouse IgG or Alexa Fluor 488 goat anti-rabbit IgG respectively). Differentiation of nuclei was achieved by staining the cell nucleus with Hoechst 33342 reagent. Labelled cells were then examined by confocal microscopy. The aim of this work was to characterise the cellular distribution of Vif protein, and to examine whether the distribution was affected when co-expressed with Triad 3au which might indicate an intracellular interaction.

Initially, to determine the distribution of Vif, 293T cells were transfected with pMyc-Vif and pHA plasmid, then stained (see section **2.2.10**). Vif stained with the polyclonal rabbit antibody, was detected in all cellular compartments (Figure 3.12A), consistent with previous reports (Chatterji et al., 2000, Goncalves et al., 1994, Goncalves et al., 1995, Simon et al., 1997). Vif exhibited a diffuse distribution in the cytoplasm and was localised in distinct foci present at the plasma membrane and also in the cell nucleus (Figure 3.12 B). Next, 293T cells were co-transfected with pMyc-Vif and pHA-Triad 3au and dually stained with anti-Vif polyclonal antibody and HA monoclonal antibody, followed by Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG secondary antibodies. Co-localisation of Vif and Triad 3au was seen in cytoplasm and at cellular membranes of co-transfected cells (Figure 3.13D, yellow areas). Also, Vif protein aggregations were clearly present on cellular membranes when co-transfected with Triad 3au (Figure 3.13D, white arrow).

Figure 3.12 Vif localization analysis

293T cells were co-transfected with pCMV-Myc-vif and pCMV-HA vector and stained with Vif rabbit polyclonal antibody and goat anti rabbit IgG labelled with Alex Fluorescence 488, followed by Hoechst 33342 to identify nuclei. A) Vif was distributed in membrane and cytoplasm (green) and B) Vif was present also in the nucleus (blue) of transfected cells

A



B

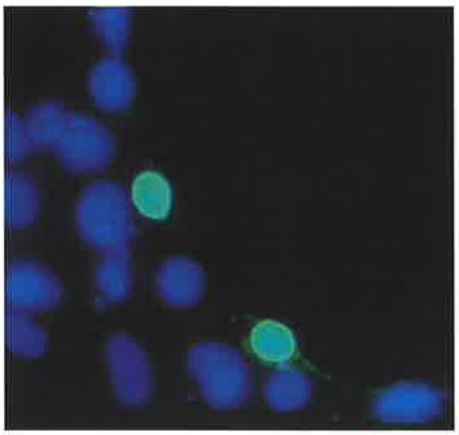


Figure 3.13 Triple labelling of pMyc-Vif and pHA-Triad 3au cotransfected 293T cells

Red: HA-Triad 3au

Primary: mouse anti HA

Secondary: Alexa Fluor 546 goat anti-mouse IgG

Green: Vif

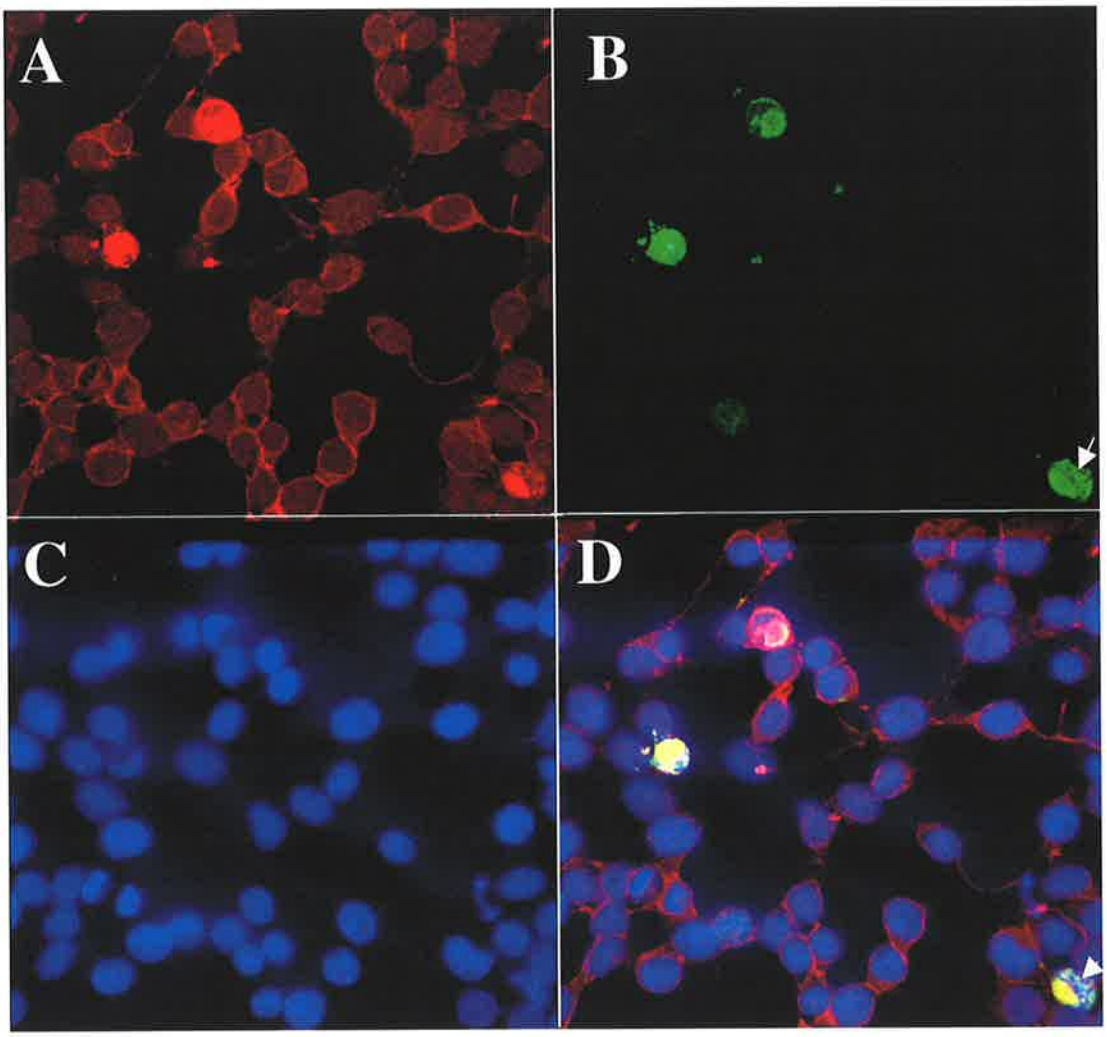
Primary: rabbit anti Vif

Secondary: Alexa Fluor 488 goat anti-rabbit IgG

Blue: Cell nuclei

Hoechst 33342

Yellow: HA-Triad 3au/Vif


A
B
C
D

Vif has been reported to localise in multiple subcellular sites including the plasma membrane (Goncalves et al., 1994, Goncalves et al., 1995, Simon et al., 1997), cytoplasm (Goncalves et al., 1994, Karczewski & Strebel, 1996), cytoskeleton (Karczewski & Strebel, 1996) and nucleus (Chatterji et al., 2000, Goncalves et al., 1995, Simon et al., 1997). Of note it has also been reported that the concentration of fixative used in the treatment of cells before staining was a factor determining the extent of nuclear staining (Thunnissen et al., 1981). Only cells fixed using a low (1-2%) percent formaldehyde exhibited nuclear staining, and this was abolished at higher concentrations of fixative. The detection of Vif in the nucleus described in this study is consistent with the mild (1% formaldehyde) fixative protocol used here (section 2.2.10). In this study, Vif was also diffusely distributed in the cytoplasm of 293T cells transfected with the pMyc-Vif and pHA plasmids and was also seen in distinct foci present at the plasma membrane. In contrast, when cells were cotransfected with pMyc-Vif and pHA-Triad 3au, the distribution of Vif was altered resulting in aggregates which co-localised with Triad 3au. This suggested that the change of Vif distribution was caused by expression of Triad 3au but not the HA epitope. These confocal images lend further support to our previous result showing co-immunoprecipitation of Vif and Triad 3au in co-transfected 293T cells.

3.5 Triad 3au mRNA is expressed in both permissive and primary non-permissive cells

The Triad 3au sequence does not start with a potential translation initiation codon ATG sequence suggesting that this sequence may represent a truncated part of a larger cellular protein in human leucocytes. TRIAD containing proteins were

identified in database searches of *Aedes aegypti* (mosquito), *D. melanogaster*, *C. elegans*, mouse and human sequences (van der Reijden et al., 1999). To determine whether a protein homologue of Triad 3au may be present in mammalian cells, the presence of Triad 3au specific mRNA in this cell type was investigated. In addition, the levels of Triad 3au specific mRNA was compared between non-permissive and permissive cells, since it has been proposed that non-permissiveness may be due to the presence of cellular inhibitor of HIV-1 infection that is overcome by Vif in non-permissive cells (Madani & Kabat, 1998, Simon et al., 1998a).

Total cell RNA was extracted from a variety of different cell types and the levels of Triad 3au mRNA estimated by Northern blot analysis as described in section **2.2.3**. RNA was extracted from 3 individual aliquots of freshly isolated primary human PBL, macrophages and other non-permissive cell lines including H9, A3.01 and HuT-78 cells, as well as from permissive cell lines 293T, CEM-ss, COS-7, and HeLa cells. RNA resolved in 1% agarose gels typically exhibited two bands at approximately 4.5 Kb and 1.9 Kb in size when examined by ethidium bromide staining, corresponding to 28S and 18S ribosomal RNA species respectively (Figure 3.14). Distinct staining of these RNAs was diagnostic of an RNA sample with limited degradation and was used to assess the quality of the RNA samples. Triad 3au mRNA was then detected by northern blot using a ^{32}P-labelled Triad 3au cDNA probe. A typical northern blot is presented in Figure 3.15 A. The relative mRNA levels were estimated by normalisation against GapDH mRNA levels detected in the same sample (Figure 3.15 B). Triad 3au mRNA was detected in all tested cell types, as two species approximately 3.0 Kb and 6.0 Kb in size. Triad 3au mRNA levels after normalisation against GapDH mRNA were slightly increased in primary PBLs

Figure 3.14 RNA analysis from ethidium bromide stained gel

Total RNA extracted from (A) Non-permissive cells: PBL, macrophage, HuT-78, A3.01, H9 (lane 1-5 respectively). (B) Permissive cells: COS-7, HeLa, 293T, CEMss (lane 1-4 respectively).

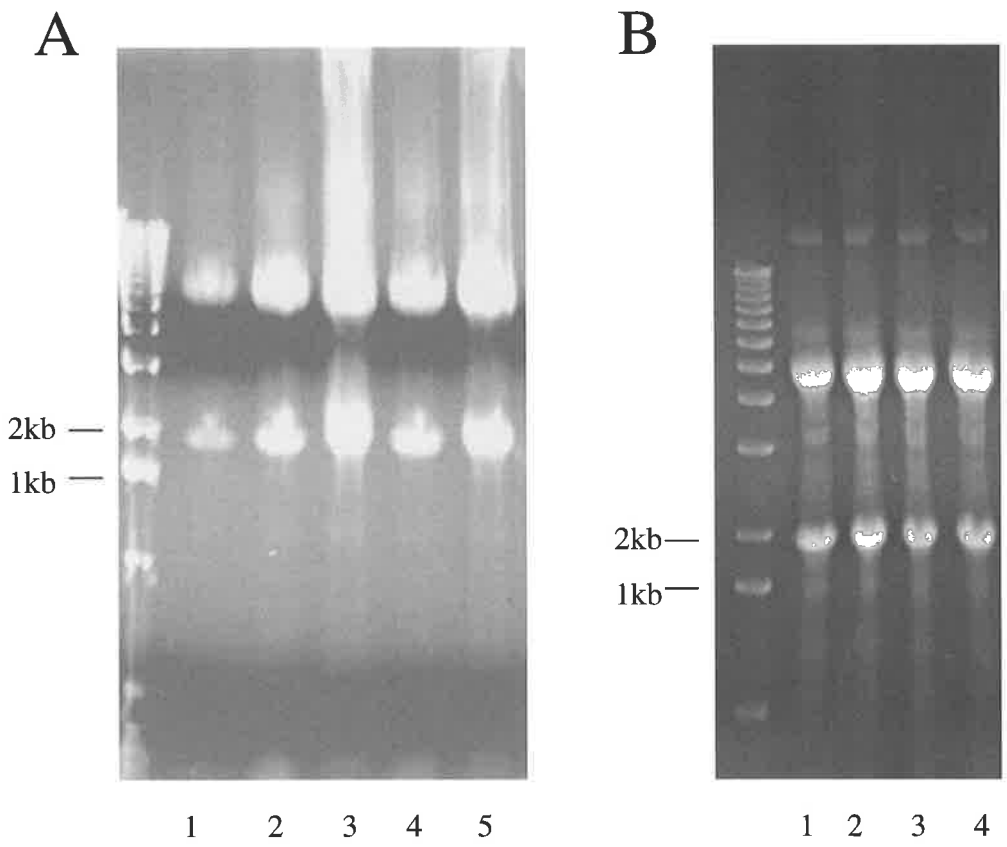
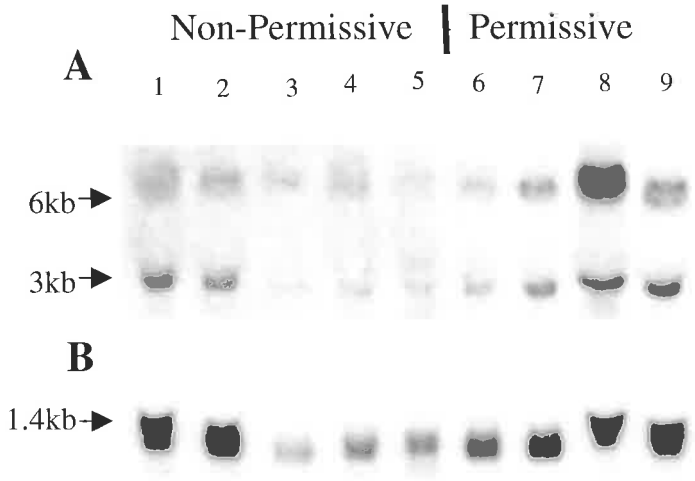

A
2kb
1kb
1 2 3 4 5
B
2kb
1kb
1 2 3 4

Figure 3.15 Triad 3au mRNA is expressed in permissive and non-permissive cells.

Northern blot hybridization of total RNA extracted from non-permissive cells HuT-78, H9, PBL, macrophages, and A3.01 (lane 1-5 respectively) and permissive cells 293T, CEM-ss, COS-7 and HeLa (lane 6-9 respectively). The filter was initially probed with ^{32}P-labeled Triad 3au cDNA (panel A), then stripped and re-probed with ^{32}P-labeled GAPDH cDNA (Panel B)


Non-Permissive
Permissive
A
1 2 3 4 5 6 7 8 9
6kb
3kb
B
1.4kb

and macrophages compared with other cell types, but the levels were similar in all cell types regardless of their permissiveness status for Δ*vif* HIV replication. These results indicated that a mRNA containing Triad 3au specific sequences was present in a variety of mammalian cells, as it was found in both in non-permissive and permissive cell types, it was unlikely to represent the above inhibitor of HIV-1 infection, at least independently. Previous yeast two-hybrid assays and immunoprecipitation and immunofluorescent studies of mammalian cells have shown that Triad 3au interacts with Vif. Therefore the natural cell protein homologue of Triad 3au was investigated, to examine whether this interaction played a role in regulating Vif function in HIV-1 infection.

3.6 Identification of ZIN and preparation of the ZIN cDNA

In May 2002, following the beginning of this study a protein homologue of the hypothetical Triad 3 protein was identified in cells by Chen *et al* and named Zinc finger protein inhibiting NF-*k*B (ZIN) based on functional studies (Chen et al., 2002). ZIN was shown to contain four RING-like zinc finger domains and a proline-rich C terminal domain. Northern blot hybridisation and western blot analysis showed that ZIN was expressed in all cell lines tested including PBL, 293, RPMI8226 and Jurkat cells. Confocal microscopy images suggested ZIN was a cytoplasmic protein that co-localised in mammalian cells with the serine / threonine protein kinase receptor interacting protein, RIP. RIP functions as an adaptor protein in a signal transduction pathway that is activated by stimulation of the TNF-α receptor and leads to activation of NF-kB. Overexpression of ZIN in 293 cells demonstrated that ZIN

interacted with RIP and inhibited RIP mediated activation of NF-*K*B that normally resulted when cells were stimulated with TNF-α (Chen et al., 2002).

Sequence alignment of Triad 3au with the new Triad 3 homologue, ZIN was performed employing the BioEdit sequence analysis program. The ZIN protein sequence was adapted from the GenBank™ database (NP_061884). The 218 amino acid Triad 3au amino acid sequence mapped almost identically with an equal 218 amino acid region of ZIN comprising the 4 ring-like domains (RLDs) but not the N- or C-terminal sequences (Figure 3.16). One amino acid difference was apparent between the two sequences with an alanine instead of a proline at position 219 (numbering based on the ZIN sequence) in Triad 3au. This single difference was confirmed in repeat sequencing experiments using the same Triad 3au clone. Therefore these results demonstrated that Triad 3au was 99.8% homologous with a segment of the Triad 3 homologue, ZIN.

In section **3.5**, it was found that Triad 3au specific mRNAs, 3.0 Kb and 6.0 Kb in size, were present in all cells types at similar levels. Consistent with this, Chen *et al* (2002) found by northern blot that a ZIN cDNA probe detected two mRNA, also 3.0 Kb and 6.0 Kb in size (Chen et al., 2002). Based on almost identical sequence homology and northern blot, data ZIN was chosen as the most likely naturally expressed candidate protein homologue of Triad 3au, and therefore became the primary focus of this study.

In order to investigate whether full-length Triad 3au or ZIN could also interact with Vif, cDNA encoding ZIN was synthesized using BD SMART^{TM} RACE cDNA

Figure 3.16 Sequence analysis of Triad 3au and ZIN.

Deduced protein sequences of the 488 amino acid ZIN (upper) and the 218 amino acid Triad 3au sequence (lower). These sequences are determined in the current study. The GenBank™ accession number for the previously published ZIN protein sequence is NP_061884. Triad 3au amino acid residues that are identical to the ZIN sequence are marked with asterisks. The two amino acid residues different from the published ZIN sequence are circled. Ring like domains (RLDs) are enclosed by open boxes. Proline rich domain (PRD) is underlined.


ZIN
MADFKVLSSQDIKWALHELKGHYAITRKALSDAIKKWQELSPETSGKRKK 50
RKQMNQYSYIDFKFEQGDIKIEKRMFFLENKRRHCRSYDRRALLPAVQQE 100
RLD1
QEFYEQKIKEMAEHEDFLLALQMNEEQYQKDGQLIECRCCYGEFPFEELT 150
Triad 3au
QCADAHLFCKECLIRYAQEAVFGSGKLELSCMEGSCTCSFPTSELEKVLP 200
RLD2
QTILYKYYERKAEEEVAAAYADELVRCPSCSFPALLDSDVKRFSCPNPHC 250
RKETCRKCQGLWKEHNGLTCEELAEKDDIKYRTSIEEKMTAARIRKCHKC 300
RLD3
RLD4
ETGLIKSEGCNRMSCRCGAQMCYLCRVSINGYDHFCQHPRSPGAPCQECS 350
RCGLWTDPTEDDEKLIEEIQKEAEEEQKRKNGENTFKRIGPPLEKPVEKV 400
QRVEALPRPVPQNLPQPQMPPYAFAHPPFPLPPVRPVFNNFPLNMGPIPA 450
PRD
PYVPPLPNVRVNYDFGPIHMPLEHNLPMHFGPQPRHRF 500

Amplification Kit (Clontech Cat # K1811-1). The strategy to generate a full-length cDNA from an RNA template is described in Figure 3.17. Firstly, RNA extracted from A3.01 cell was treated with DNase, and first strand cDNA synthesis was then initiated by BD PowerScript^{TM} Reverse Transcriptase (RT). BD PowerScript RT is a variant of Moloney Murine Leukemia Virus (MMLV) RT that, upon reaching the end of an RNA template, exhibits terminal transferase activity, adding 3–5 residues (predominantly dC) to the 3' end of the first-strand cDNA. The BD SMART oligo contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT. BD PowerScript RT switches templates from the mRNA molecule to the BD SMART oligo, generating a complete cDNA copy of the original RNA with the additional BD SMART sequence at the end. Completed ZIN cDNA was then amplified by PCR using ZIN specific 5' and 3' sequence primers complementary to the published ZIN mRNA GenBank™ sequence (AY063174). The cDNA product of this reaction was separated on a 1% agarose gel and confirmed as a single DNA product approximately 1.5 Kb in size as expected from the ZIN sequence (Figure 3.18). The PCR product was excised from the gel and cloned into the pCMV-Flag protein expression vector, and the construct was confirmed by restriction digest analysis using *EcoRI* and *BamHI*. Sequence analysis of the cloned cDNA sequence also confirmed that it encoded the complete 488 amino acid ZIN protein except for a single amino acid leucine to phenylalanine change at position 30. This may have arisen because of an error in the reported ZIN sequence, as the sequence of our isolate was confirmed using several independently generated PCR products (Figure 3.19). The significance of the single amino acid difference is not known, but in any case it would not be expected to alter ZIN function as it is

Figure 3.17 Strategy for first strand cDNA synthesis

Adapted from Clontech, Protocol No. PT3269-1

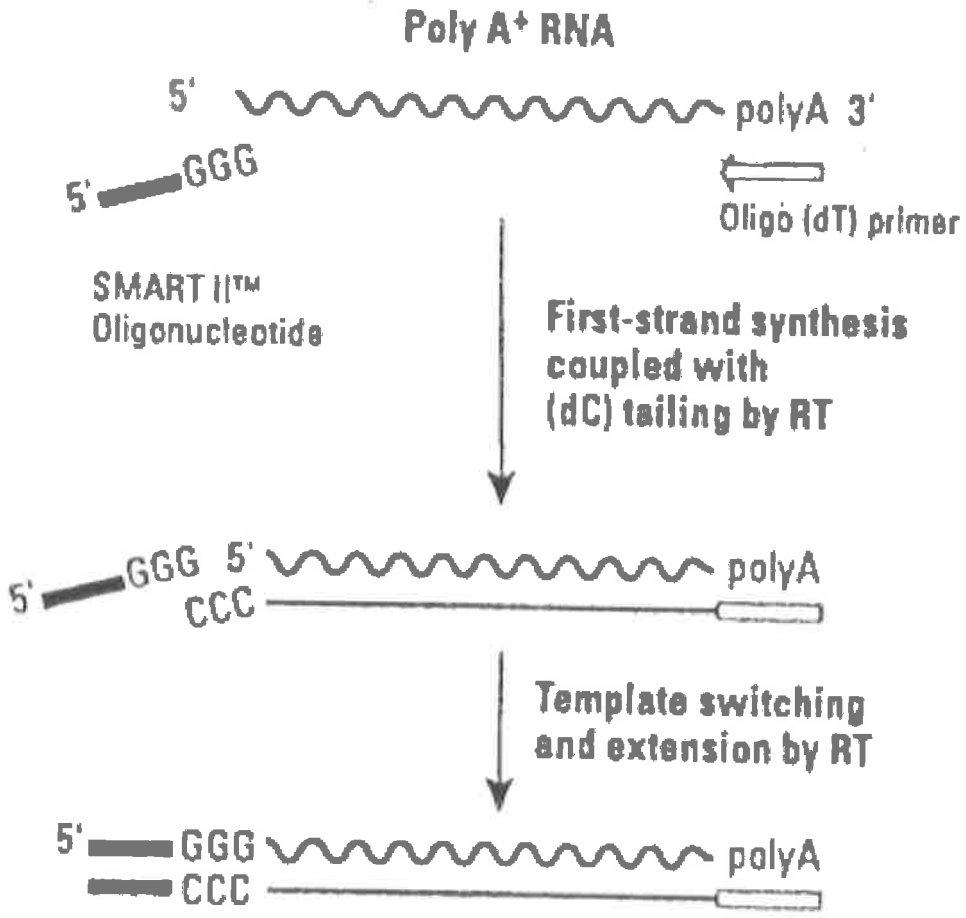

Poly A+ RNA
5'
polyA 3'
5'
GGG
Oligo (dT) primer
SMART II™
Oligonucleotide
First-strand synthesis
coupled with
(dC) tailing by RT
5'
GGG
5'
polyA
CCC
Template switching
and extension by RT
5'
GGG
polyA
CCC

Figure 3.18 Full-length ZIN cDNA synthesis

Lane 1 & 2 indicate the PCR product amplified from total RNA extracted from A3.01 cells. Lane 3 & 4 show the PCR product amplified from total RNA extracted from human placental cells (positive control provided by Clontech). + RTase and - RTase indicate that RNA was or was not treated respectively, with BD PowerScript™ Reverse Transcriptase (Clontech).

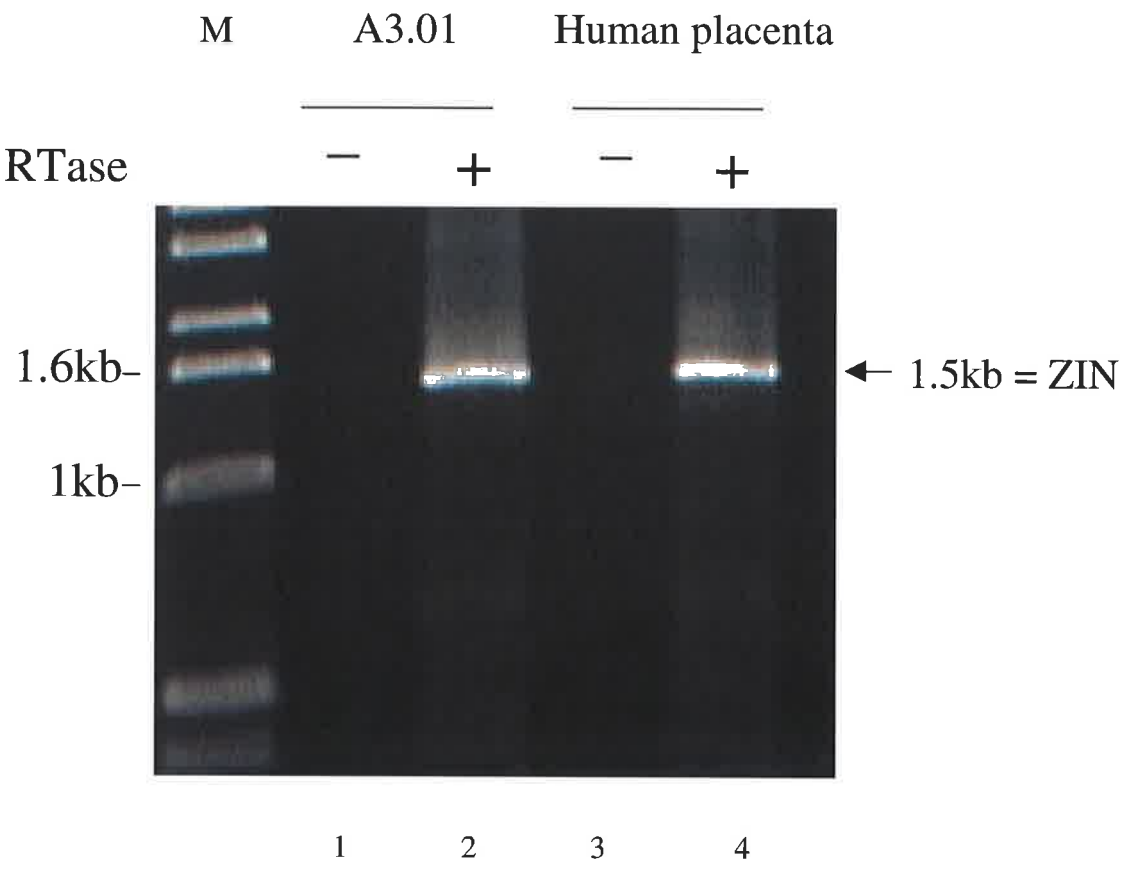

M
A3.01
Human placenta
RTase
−
+
−
+
1.6kb–
1kb–
← 1.5kb = ZIN
1
2
3
4

Figure 3.19 Sequence comparison of newly isolated ZIN and reported ZIN

Deduced protein sequences of newly isolated ZIN cDNA prepared in this study (termed ZIN A3.01), and the reported ZIN sequence (termed ZIN) (GenBank™ accession number NP-061884). Black dots indicate identical amino acid residues in both ZIN sequences.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        10         20         30         40         50
ZIN              MADFKVLSSQ DIKWALHELK GHYAITRKAL SDAIKKWQEL SPETSGKRKK
ZIN(A3.01)       .......... .......... .........F .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
ZIN              RKQMNQYSYI DFKFEQGDIK IEKRMFFLEN KRRHCRSYDR RALLPAVQQE
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
ZIN              QEFYEQKIKE MAEHEDFLLA LQMNEEQYQK DGQLIECRCC YGEFPFEELT
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
ZIN              QCADAHLFCK ECLIRYAQEA VFGSGKLELS CMEGSCTCSF PTSELEKVLP
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
ZIN              QTILYKYYER KAEEEVAAAY ADELVRCPSC SFPALLDSDV KRFSCPNPHC
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
ZIN              RKETCRKCQG LWKEHNGLTC EELAEKDDIK YRTSIEEKMT AARIRKCHKC
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350
ZIN              GTGLIKSEGC NRMSCRCGAQ MCYLCRVSIN GYDHFCQHPR SPGAPCQECS
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       360        370        380        390        400
ZIN              RCSLWTDPTE DDEKLIEEIQ KEAEEEQKRK NGENTFKRIG PPLEKPVEKV
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                       410        420        430        440        450
ZIN              QRVEALPRPV PQNLPQPQMP PYAFAHPPFP LPPVRPVFNN FPLNMGPIPA
ZIN(A3.01)       .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|...
                       460        470        480
ZIN              PYVPPLPNVR VNYDFGPIHM PLEHNLPMHF GPQPRHRF
ZIN(A3.01)       .......... .......... .......... ........
```

located outside the RLDs. pCMV-Flag-ZIN (pFlag-ZIN) was used in subsequent experiments.

Chapter 4

Interaction between HIV-1 Vif and ZIN, and Implications for Viral Replication

4.1. Introduction

As previously described, Triad 3au was isolated from a human leukocyte cDNA library and sequence analysis suggested that it represented part of a larger uncharacterized, hypothetical protein called Triad 3. Subsequent northern blot analysis of RNA extracted from a variety of cell types identified two Triad 3au specific RNAs 3.0 Kb and 6.0 Kb RNA in size. More recently, Chen *et al*, 2002 identified a larger sequence ZIN from a human B cell cDNA library and also detected a 3.0 Kb and 6.0 Kb RNA species by northern blot in different human tissues and PBL cell. The C-terminal sequence of ZIN was almost identical homology to Triad 3 (Chen et al., 2002). Based on 99.8% homology between the 218 amino acid Triad 3au protein sequence and a 218 amino acid stretch of 488 amino acid ZIN protein (Figure 3.16; 142 aa –359 aa), ZIN was considered the most likely cell protein homologue for Triad 3au.

In TNF-α stimulated human embryonic kidney 293 cells that are over-expressing ZIN, ZIN inhibits NF-*K*B activation by interfering with the induced signal transduction pathway through its interaction with the TNF-α receptor adapter protein, RIP (Chen et al., 2002). Overexpression of ZIN also potentiates RIP- and TNF-induced apoptosis in these same cells in a dose dependent manner. What other functions ZIN may play in these cells or others, including B and T lymphoma cells

where ZIN is also expressed naturally (Chen et al., 2002), is not known. In light of the activation between Triad 3au and HIV Vif (Chapter 3), it was therefore of interest to examine firstly whether ZIN also interacted with HIV-1 Vif protein and secondly, if so, could this be of significance for Vif function and HIV replication.

4.2 ZIN interacts with purified Vif *in vitro*

The GST pull-down assay is a biochemical method for demonstrating *in vitro* protein-protein interactions qualitatively. A commercially available GST system has been widely used (Figure 4.1). Briefly, a 'target' gene encoding a protein of interest (in this case Vif) is joined to the glutathione S-transferase (GST) gene and expressed in *E. coli,* to synthesise a GST fusion protein that is then purified and immobilised on glutathione-Sepharose CL-4B beads. Candidate proteins (in this case ZIN) are incubated with the beads, and material that remains bound to the GST-CL-4B beads by virtue of its interaction with the 'target' protein is then identified by western blot

The GST-Vif fusion protein and GST alone were expressed in *E.coli* cells transformed with pGEX-2T-GST-Vif (pGST-Vif) and pGEX-2T-GST (pGST) respectively (section **2.2.11**). The level of pGST-Vif protein expressed was normalised against serially diluted GST protein on SDS-PAGE gels stained with coommassie-blue (Figure 4.2). Lane 1 and lane 5 of figure 4.2 show similar protein levels in the 1:50 times dilution of GST protein and the same volume of neat GST-Vif protein respectively. Thus, equal amounts (~ 2 μg total protein) of 1:50 - diluted GST protein and neat GST-Vif protein were incubated with Glutathione Sepharose CL-4B beads and the protein-GST-bead complex finally thoroughly washed free of non-specific bacterial lysate proteins. ZIN containing cell lysate, for use in the GST

Figure 4.1 Principle of GST pull-down assay

GST-Vif is expressed in BL21 cells, ZIN is produced in 293T cells. Following the steps below

Step 1: GST-Vif is coupled to GST-CL-4B beads

Step 2: Beads bound with either GST-Vif or GST are incubated with ZIN

Step 3: Beads washed and bound protein detected by western blot

If ZIN interacts with Vif, ZIN can be isolated by its attachment to the beads

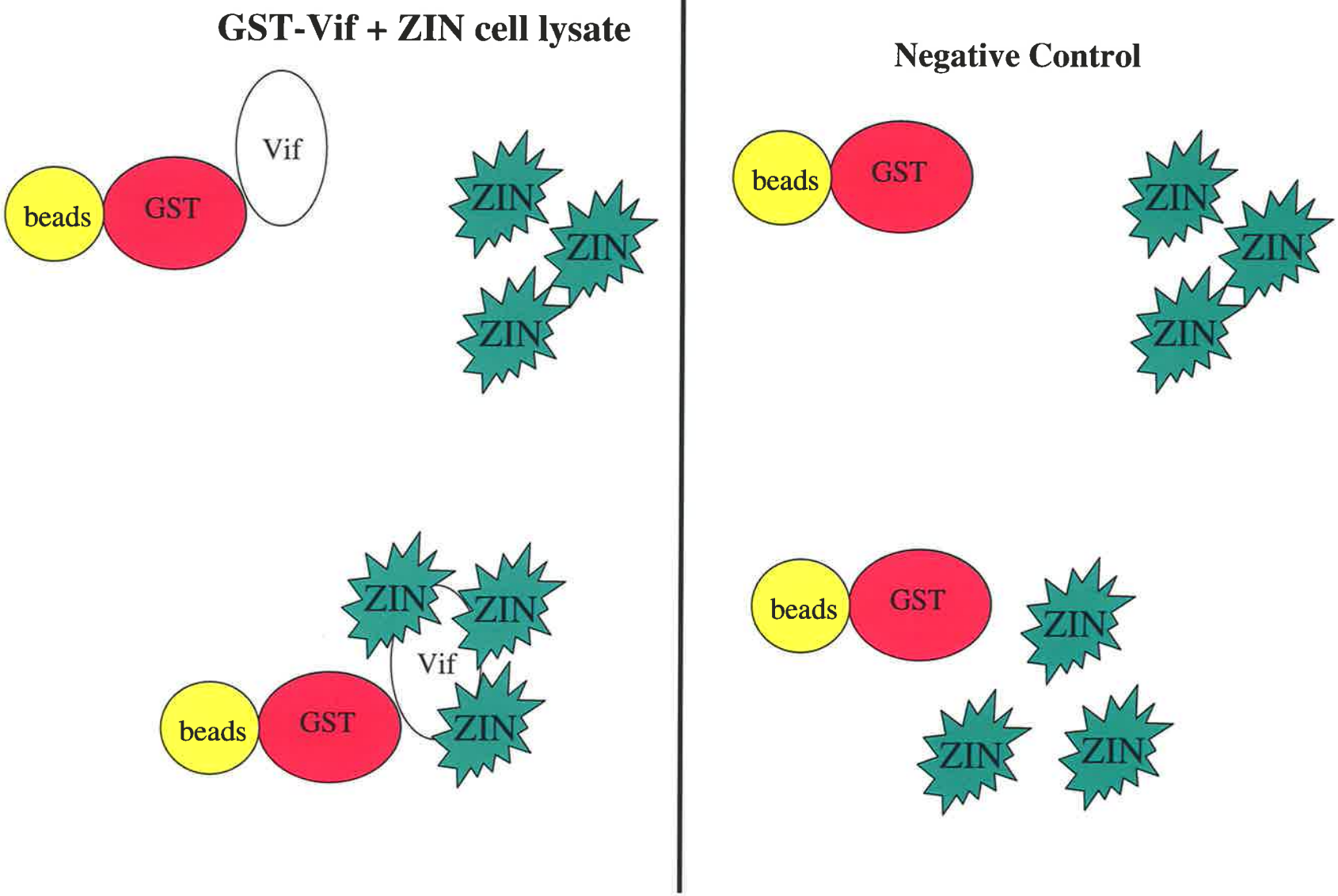
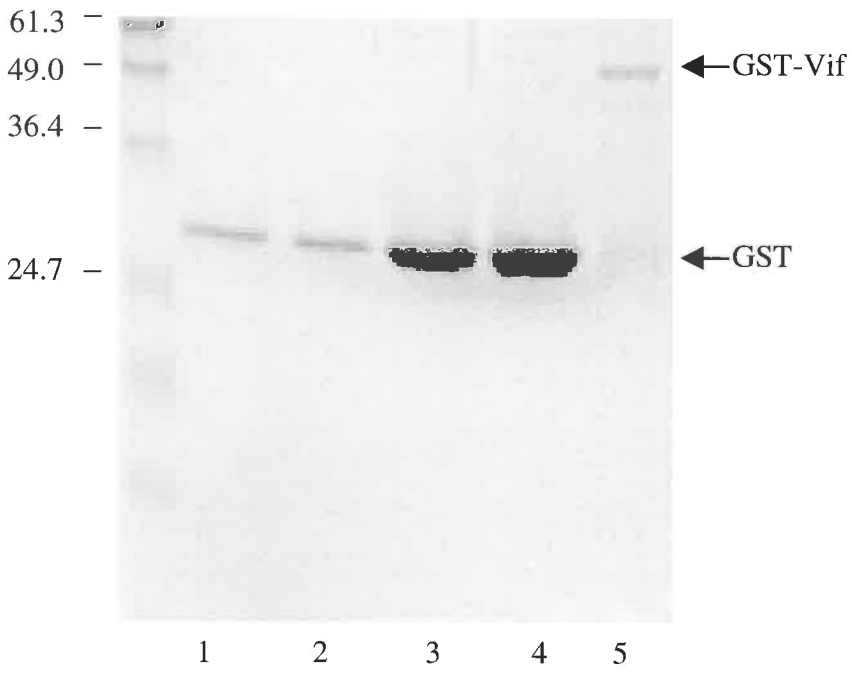

GST-Vif + ZIN cell lysate
Vif
beads
GST
ZIN
ZIN
ZIN
ZIN
ZIN
Vif
beads
GST
ZIN
Negative Control
beads
GST
ZIN
ZIN
ZIN
beads
GST
ZIN
ZIN
ZIN

Figure 4.2 Normalisation of GST-Vif fusion protein against GST protein by coommassie-blue staining

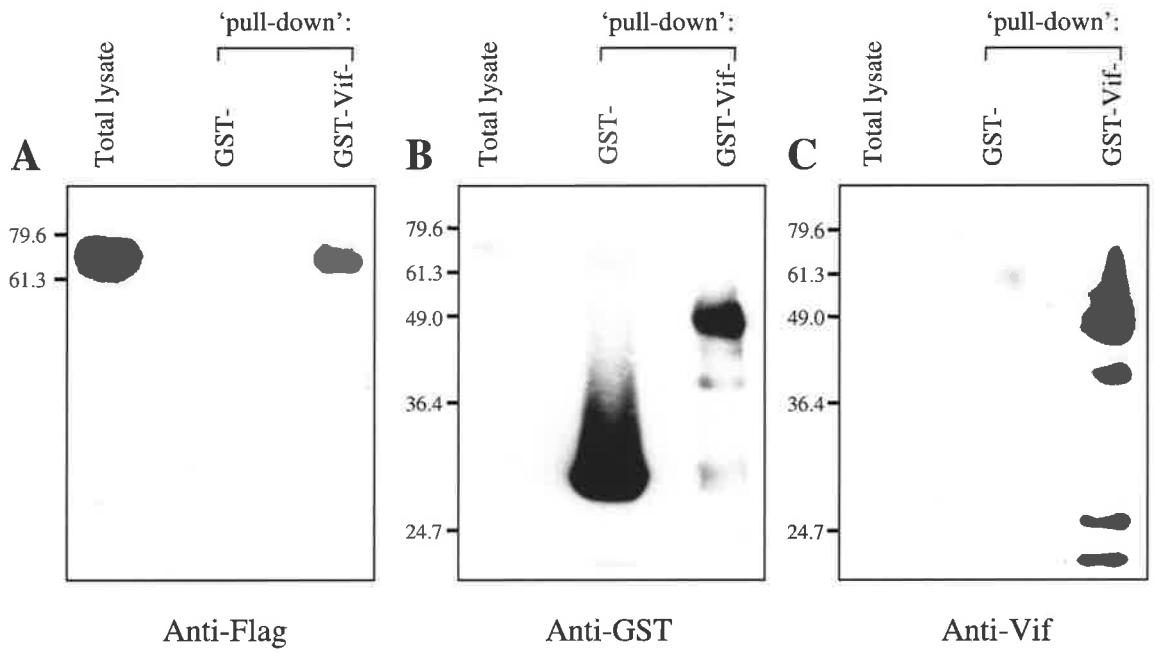
Total GST-Vif fusion protein was loaded in lane 5 (49.0kd), GST alone (26kd) protein for negative control was serially diluted at 1:50 (lane 1), 1:20 (lane 2), 1:10 (lane 3) and1:5 (lane 4)


61.3
49.0
36.4
24.7
GST-Vif
GST
1
2
3
4
5

fusion protein pull–down assay, was produced by transfection of pFlag-ZIN in 293T cells using the SuperFect method (see section **2.2.11**). Finally the GST (or GST-Vif) complexed glutathione sepharose CL-4B beads were incubated with Flag-ZIN, pelleted and analysed by western blot using antibodies specific for Flag fusion protein, GST and Vif. Using murine Flag specific antibody, Flag-ZIN was detected in the total 293T cell lysate as a 61 kDa protein product (Figure 4.3A, lane 1). Flag-ZIN was also detected in protein 'pulled – down' by GST-Vif / glutathione sepharose CL-4B beads (Figure 4.3.A, lane 3), but not by glutathione sepharose CL-4B beads complexed with GST alone (Figure 4.3A, lane 2). Hence co-precipitation of Flag-ZIN with glutathione sepharose CL-4B beads was dependent on Vif in the Vif-GST fusion protein. The Western blots were then stripped of antibody and reprobed with GST specific antibody to confirm that equal amounts of glutathione sepharose CL-4B beads complexed with either GST or GST-Vif were used in each 'pull-down' sample. A GST-Vif fusion protein of approximately 49 kDa was detected in protein precipitate that 'pulled –down' Flag-ZIN (Figure 4.3B, lane 3), and as expected the 26 kDa GST protein was also readily detected in sample containing GST / glutathione sepharose CL-4B beads alone (Figure 4.3B, lane 2). This indicates that 'pull-down' of flag-ZIN by GST-Vif / glutathione sepharose CL-4B beads and not GST / glutathione sepharose CL-4B beads was dependent on the presence of Vif and not the result of a non-specific interaction between Flag-ZIN and GST protein. The identity of the 49 kDa GST-Vif fusion protein responsible for 'pull-down' of Flag-ZIN was also confirmed by stripping and reprobing the filter with Vif specific antibody. The polyclonal anti-Vif antibody reacted with a 49 kDa GST-Vif fusion protein and several bands with lower molecular weights, probably representing Vif

Figure 4.3 Vif directly binds ZIN in vitro.

Western blot analysis of total protein (lane 1) and protein precipitated from 293T cell lysates expressing Flag-ZIN fusion protein by incubation with glutathione sepharose CL-4B complexed proteins, GST (lane 2) and GST-Vif (lane 3). Blots were probed with (A) monoclonal anti-Flag antibody, (B) anti-GST antibody and (C) polyclonal anti-Vif antiserum.


A
B
C
Total lysate
'pull-down':
GST-
GST-Vif-
79.6
61.3
49.0
36.4
24.7
Anti-Flag
Anti-GST
Anti-Vif

cleaved from GST-Vif fusion protein or minor proteolytic products brought about by protease activity in the *E.coli* lysate preparation. (Figure 4.3 C, lane 3).

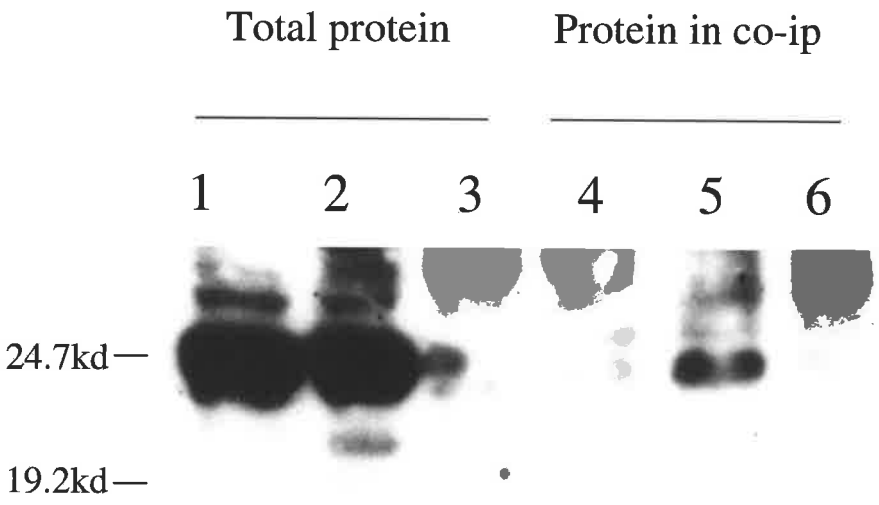
Earlier attempts at this 'pull-down' experiment resulted in 'pull-down' of Flag-ZIN from cell lysate, by both GST-Vif / glutathione sepharose CL-4B beads and to a lesser extent GST / glutathione sepharose CL-4B beads as well (Figure not shown). To avoid non-specific protein-protein interactions, precleared lysate from Flag-ZIN transfected 293T cells (supernatant, 13,500 rpm, 4°C for 10 min, followed by treatment with glutathione sepharose CL-4B beads) was then incubated with GST / glutathione sepharose CL-4B beads at 4°C for 1 hr (see section **2.2.11**). The mixture was spun by low speed centrifugation to remove the added GST / glutathione sepharose CL-4B beads and any non-specifically bound lysate proteins. Experiments as shown in Figure 4.3 were then undertaken 3 times with consistent results, demonstrating a specific interaction between ZIN and Vif.

4.3 ZIN interacts with HIV-1 Vif in mammalian cells

To determine whether the above interaction also occurred in mammalian cells, co-immunoprecipitation experiments were performed. Here, 293T cells were transfected as described in section **2.2.7**, either with pMyc-Vif together with pFlag-ZIN, or with pMyc-Vif and pFlag as a negative control. Cell lysates were then incubated with Flag specific monoclonal antibody which was then immunoprecipitated using protein A sepharose beads (section **2.2.9**). Any Myc-Vif fusion protein that may have been co-precipitated by the Flag specific monoclonal antibody was then detected by western blot using rabbit anti-Vif antiserum. Despite abundant expression of Myc-Vif protein in both transfected cell cultures (Figure 4.4, lanes 1 and 2), Myc-Vif was only

Figure 4.4 Co-immunoprecipitation of Vif with ZIN.

293T cells co-transfected with pMyc-Vif and pFlag (lanes 1, 4), pMyc-Vif and pFlag-ZIN (lanes 2, 5), or no DNA (lanes 3,6), were analysed by Western blot probed with polyclonal Vif antiserum. Lanes 1, 2, 3 represent total protein in the lysates before immunoprecipitation, and Lane 4, 5, 6 represent protein immunoprecipitated with mouse anti-Flag antibody on protein A sepharose beads.


Total protein
Protein in co-ip
1
2
3
4
5
6
24.7kd
19.2kd

detected in immunoprecipitate containing Flag-ZIN protein (Figure 4.4, lane 5), and not in that containing Flag protein alone (Figure 4.4, lane 4). This indicated that Vif was co-immunoprecipitated because of a specific interaction with ZIN rather than the Flag epitope. This result clearly demonstrated that the specific interaction between ZIN and Vif characterised *in vitro* also occurred in human cells.

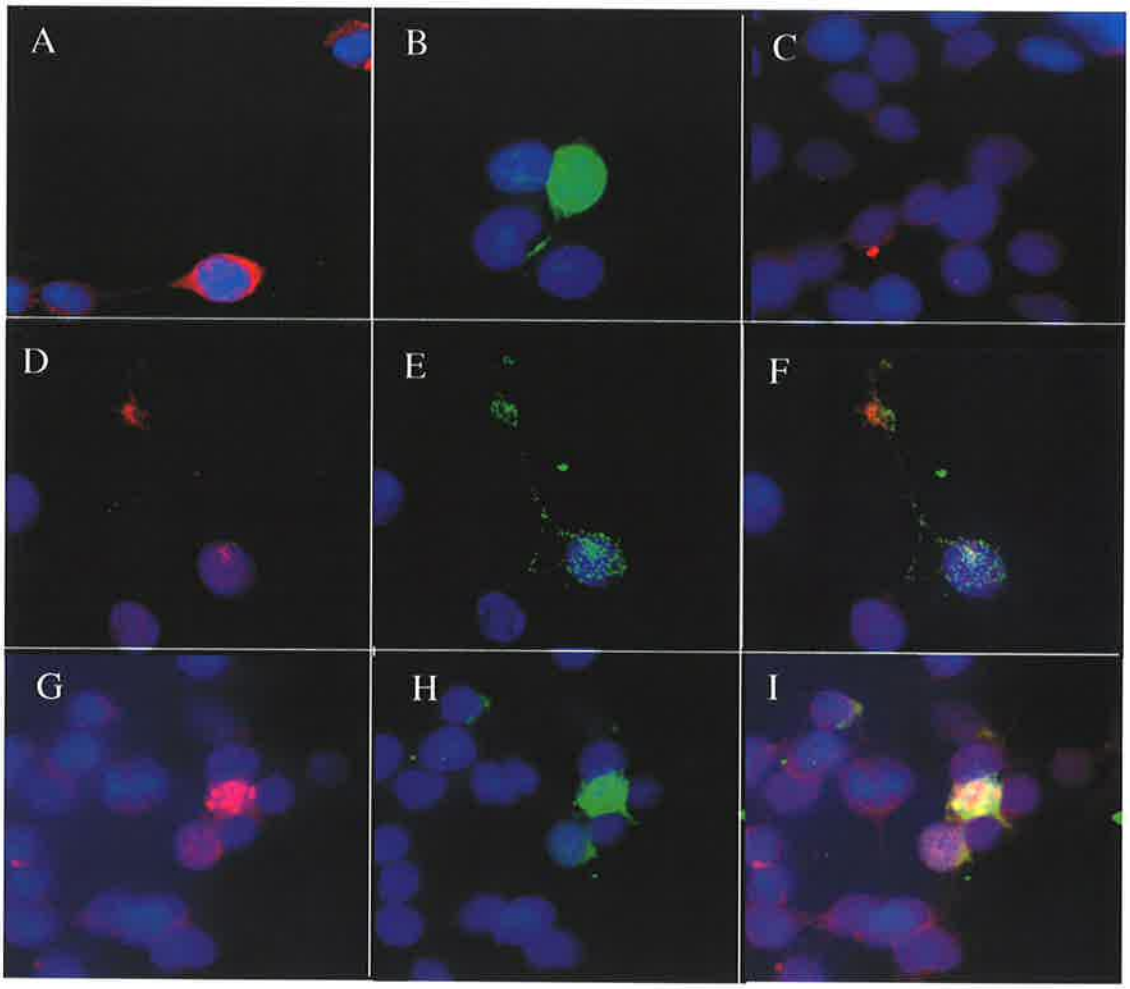
Taken together with Chapter 3, these results demonstrated that the interaction between Vif and the partial protein Triad 3au also occurs between Vif and the complete protein, ZIN. Conversely these results indicate that the central domain of ZIN shared by Triad 3au is the essential binding domain involved in the interaction with HIV-1 Vif.

4.4 Co-localisation of Vif and ZIN in transfected cells

Using the method described in section **3.4**, the cellular distribution of HIV Vif and ZIN was examined in different experimental settings. 293T cells were co-transfected with pMyc-Vif and pFlag-ZIN, or transfected with one of the above plasmids together with the control plasmid expressing alternative marker protein only (eg pMyc or pFlag) but without Vif or ZIN. The transfected cells were stained and examined by confocal microscopy. Staining of pMyc-Vif and pFlag co-transfected cells with rabbit anti-Vif antibody, followed by the secondary Alexa Fluor 488 goat anti-rabbit IgG, showed a diffuse distribution of Vif (green emission) in the cytoplasm and nucleus, at the cell membrane and along cellular extensions that characterise growing 293T cells (Figure 4.5 B). Cells cotransfected with pFlag-ZIN and pMyc, but stained with primary Flag monoclonal antibody and secondary Alexa Fluor 546 goat anti-mouse IgG, detected Flag-ZIN (red emission) localised mainly at

Figure 4.5 Co-localization of Vif and ZIN in human cells.

293T cells transfected with pFlag-ZIN plus control plasmid pMyc(A), pMyc-Vif plus control plasmid pFlag (B), or mock-transfected (C), or co-transfected with pMyc-Vif & pFlag-ZIN(D-I) were grown and fixed on glass coverslips and either non-permeabilised (D-F) or permeabilised (A-C & G-I), as described in 2.2.10. Cells were incubated with mouse anti-Flag and then Alexa Fluor 546 conjugated secondary antibodies (red emission) for detection of Flag-ZIN (A,D,G,C,F,I); or with rabbit anti-Vif antibody and then Alexa Fluor 488 goat anti rabbit IgG (green emission) for detection of Vif (C,F,I/B,E,H). Dual stained cells(C,F,I) were viewed as merged images of Alexa Fluor 488 and nuclei were stained in all panels using Hoechst 33342 (blue emission). Images were produced using the BioRad Radiance 2100 confocal microscope (BioRad Microsciences) equipped with three lasers, Argon ion 488nm (14mw), Green HeNe 543nm (1.5mw), Red Diode 637nm (5mw) outputs.


A
B
C
D
E
F
G
H
I

the cell membrane and cytoplasm (Figure 4.5A). In pMyc-Vif and pFlag-ZIN co-transfected cells, however, pMyc-Vif aggregations were observed in nucleus (Figure 4.5 H, green emission) and Flag-ZIN was also detected in foci within cell nucleus (Figure 4.5 G, red emission). Merged images of dual-stained cells co-transfected with pMyc-Vif and pFlag-ZIN showed the two proteins mainly coincident in the nuclei of these cells (Figure 4.5 I yellow emission). Taking Z-sections of dual-stained cells confirmed that the co-localised Vif and ZIN proteins were often distributed within polar regions of the cell membrane, as can be seen in Figure 4.5 F & I; and within foci present in the cell nucleus as can be seen in Figure 4.5 I. In cells transfected individually with vectors expressing protein tags alone (pMyc or pFlag), or cells co-transfected with pMyc and pFlag (mock transfected cells), staining with both Vif polyclonal and Flag monoclonal antibodies was very weak and not able to be meaningfully interpreted. Figure 4.5 C shows the pMyc and pFlag transfected 293T stained with the combined antibodies described above.

While Flag-ZIN was readily detected in the nuclei of cells transfected with pFlag-ZIN and pMyc-Vif, the lack of detection of Flag-ZIN in the nuclei of cells transfected with pFlag-ZIN and pMyc alone indicated that nuclear localization of ZIN was dependent upon its interaction with Vif in cells expressing both proteins (compare Figure 4.5A with Figure 4.5G). Vif aggregations occurred in the nuclei of cells cotransfected with pFlag-ZIN and pMyc-Vif (Figure 4.5 H) but was not observed in cells transfected with pFlag and pMyc-Vif (Figure 4.5 B), also suggesting that ZIN but not Flag altered the distribution of Vif. Thus, the intracellular distribution of ZIN and Vif was different in cotransfected cells from that seen in cells singly transfected with either component. This is consistent with the interpretation that the ZIN/Vif interaction demonstrated above is modifying the

intracellular localisation of both proteins. In some cells co-transfected with pMyc-Vif and pFlag-ZIN that had not been permeabilised to antibodies, weak co-staining of Vif and Flag-ZIN was seen at the extending cytoplasmic membrane that may suggest co-localization of Vif/Flag-ZIN at the surface of cell membrane (Figure 4.5.F). In the majority of non-permeabilised cells however, there was no evidence of Vif and Flag-ZIN co-staining (data not shown). This may also suggest that occasional membrane co-localization of the two proteins in co-transfected cells most likely occurred at the intracellular leaflet of the cell membrane.

Similar experiments with cells co-transfected with pMyc-Vif and pHA-Triad 3au were also described in Chapter 3. Triad 3au co-localized with Vif in the cytoplasm and at the cell membrane of co-transfected cells (Figure 3.13). However, Triad 3au in the presence of Vif was not detected in the nuclei of the co-transfected cells. This result was different from the above finding that Vif and ZIN co-localised mainly in cell nuclei. A possible explanation for this discrepancy is that full-length ZIN contains complete N-terminal and C-terminal proline-rich sequences that may modify protein structural conformation thereby causing a different protein distribution, since proline-rich regions have been shown to be important for protein multimerization (Yang et al., 2001). Proline-rich domains are also well known to interact with other domains including the Src homology 3 (SH3) domains (Hassaine et al., 2001, Singer et al., 2004). Furthermore, this interaction has been demonstrated to be implicated in the protein localisation and function (Singer et al., 2004). Thus, it is not unexpected that ZIN may have some additional localisation properties not shared by Triad 3au. The functional implications of this are not clear at present.

4.5 Effect of ZIN overexpression on production and infectivity of HIV-1

Given that ZIN interacts with HIV-1 Vif in transfected cells, the biological significance of this interaction for HIV-1 replication was examined. Human 293T cells were co-transfected with plasmids HIV-1 pNL4.3 and pFlag-ZIN (or control pFlag plasmid), or pNL4.3 (Δ*vif*) and pFlag-ZIN (or control plasmid pFlag). Cell culture media from the four transfected cultures were harvested 48 hr post infection and their p24 levels were quantified (see **2.2.12**). The results indicated that the amounts of wild-type HIV-1 or Δ*vif* HIV-1 virus produced in producer cells (293T cells) were similar regardless of the presence or absence of overexpressed ZIN. The virion infectivity relative to p24 content for each inoculum was then determined in HuT-78 cells and HeLa-CD4-β-gal indicator cells.

4.5.1 $TCID_{50}$ analysis of wild type HIV produced in the presence or absence of co-transfected ZIN

The virion infectivity titres in the culture supernatants were first measured by $TCID_{50}$ titration. HuT-78 cells were infected with 10 fold dilutions of 400 ng/ml p24 of virus in cell culture medium produced from 293T cells transfected with pNL4.3 and pFlag-ZIN (or pFlag control plasmid) in 6 replicate wells per dilution (see section **2.2.14**). Infected cell cultures were then cultured for 6 days to permit virus replication and production of virus. Using the ELISA method described in section **2.2.12**, HIV p24 levels produced 6 days post infection were then measured for each well and scored positive or negative for p24 detection. The viral titre, expressed as $TCID_{50}$ per ml of the virus inoculum, was then calculated according to the method described in section **2.2.14**. Results from 3 independent experiments indicate that NL4.3 virus produced

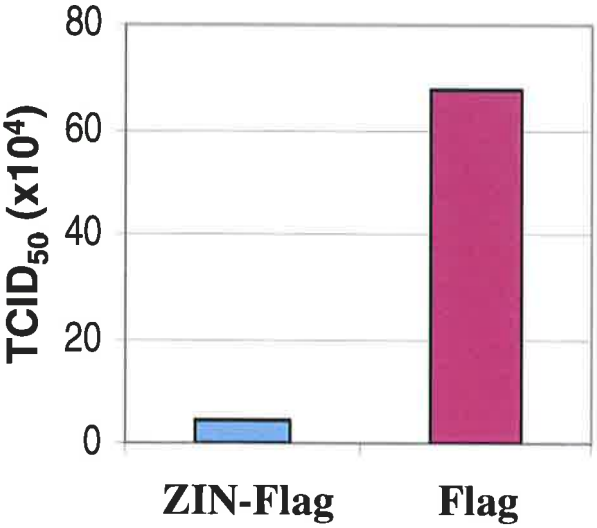
in 293T cells co-transfected with pFlag-ZIN were consistently less infectious, demonstrated by a 5-10 fold decrease in $TCID_{50}$ titre compared with NL4.3 virus produced from 293 T cells expressing only the control plasmid, pFlag. $TCID_{50}$ estimations, however, calculated from independent infection experiments were varied. These differences in the absolute calculated $TCID_{50}$ values may reflect varying growth conditions of the cells at the time of infection, slight differences in infectivity efficiencies or growth state of the HuT-78 target cells used from experiment to experiment. For this reason, absolute $TCID_{50}$ values between experiments were not compared but rather $TCID_{50}$ values within each experiment since all demonstrated the same trend. Figure 4.6 represents one result of three infection experiments. Results from this experiment indicated that NL4.3 virus produced in the presence of pFlag-ZIN was 10 fold less infectious than NL4.3 virus produced in the absence of pFlag-ZIN. Further, this difference ranged between 5 and 10 fold considering all three experimental results. Consistently then, these results suggested that overexpressed ZIN in virus producer cells had an inhibitory effect on HIV infectivity, measured by $TCID_{50}$.

4.5.2 Single-Cycle Viral Infectivity assay of wild type and Δ*vif* HIV in the presence or absence of co-transfected ZIN

The virion infectivity titre experiment showed that overexpressed ZIN decreased the infectivity of wild-type HIV. However whether ZIN mediated this effect through an interaction with Vif could not be controlled for using the same infection system because *vif* deficient HIV (pNL4.3 (Δ*vif*)) can not replicate in non-permissive HuT-78 cells. Hence to further characterise the effect of ZIN on infectivity, a single-cycle viral infectivity assay was set up using HeLa-CD4-β-gal indicator cells. This cell line

Figure 4.6 ZIN expression reduces HIV-1 infectivity

This figure shows one result of three individual infection experiments. The left bar represents the $TCID_{50}$ of NL4.3 produced in producer cells with the presence of pFlag-ZIN. The right bar indicates the $TCID_{50}$ of NL4.3 produced without the presence of pFlag-ZIN.

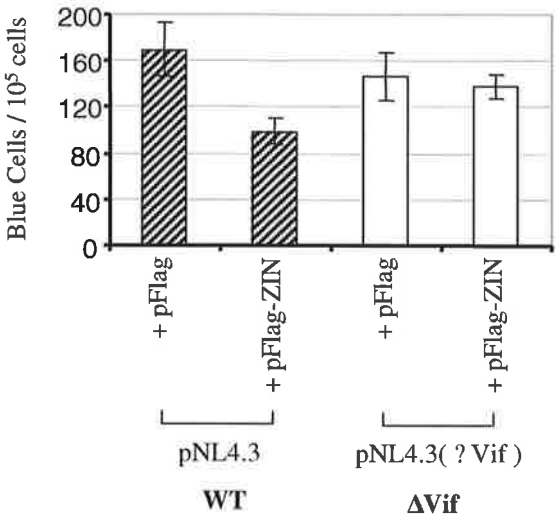

80
60
40
20
0
TCID50 (x10^4)
ZIN-Flag
Flag

expresses high level of CD4 and contains a single integrated copy of a β-galactosidase gene that is under the control of a truncated human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). This system has been used for accurate determination of viral infectivity as an alterative to end point dilution (Kimpton & Emerman, 1992). This cell line was used to quantitate the titres of HIV-1 produced in 293T cells in the presence of overexpressed Flag or Flag-ZIN.

To perform this experiment, 10-fold dilutions of 400 ng/ml p24 of each virus stock were used to infect HeLa-CD4-β-gal indicator cells. To minimise secondary infection and statistical error, cells were fixed and stained 30 hr post infection and blue cells or colonies were scored but one colony was counted as one blue cell (see section **2.2.13)**. For wells infected with neat virus, the densities of blue cells were too high to count. Results analysed from 9 replica wells (3 wells of each dilution from 10^{-1}-10^{-3}) showed that the number of blue cell or colonies obtained with wild-type HIV NL4.3 produced in the presence of co-transfected ZIN (Figure 4.7, shaded box with +pFlag-ZIN) was 2-fold less than the number of blue colonies obtained with wild-type NL4.3 but produced in the presence of Flag alone (Figure 4.7 shaded box with +pFlag). The 2 fold difference in infectivity was considered significant as it was based on counting 9 replica wells for infected (blue) cells at 30 hr pi, without resorting to multi-round viral replication. In contrast, in a similar experiment with Δ*vif* HIV NL4.3, virus produced in the presence of overexpressed ZIN (Figure 4.7, unshaded boxes with +pFlag-ZIN) or without ZIN (Figure 4.7, unshaded boxes with +pFlag) showed very similar infectivity. These results therefore suggested that 1) overexpressed ZIN but not Flag epitope in producer cells decreased the NL4.3 infectivity two-fold; 2) this effect of ZIN on virus infectivity required the presence of wild type Vif in virus producer cells.

Figure 4.7 Vif produced in cells over-expressing ZIN has reduced single-cycle infectivity

HIV virus stocks were produced in 293T cells co-transfected with pNL4.3 or pNL4.3 (Δvif) and either pFlag or pFlag-ZIN. Each of the four virus stocks was adjusted to 400 ng/ml of p24, and HeLa-CD4-LTR-β-gal cells were infected with 100ul inoculum made of 10-fold dilutions of each of the four virus stocks, in 48-well plates. At 30 hr pi cells were fixed and stained as described. Blue cells were counted in each well infected at virus dilution of 10^{-2} (0.4 ng p24/well) in which there were a minimum of 80 blue cells. Error bars were obtained from infections performed in 9 replica wells indicating the standard deviation for these measurements.


Blue Cells / 10^5 cells
200
160
120
80
40
0
+ pFlag
+ pFlag-ZIN
+ pFlag
+ pFlag-ZIN
pNL4.3
WT
pNL4.3(? Vif)
ΔVif

4.5.3 Real Time PCR analysis of viral DNA synthesis after infection

Traditional methods use Agarose gels for detection of PCR products at the final end-point of the PCR reaction. Real-Time PCR or Quantitative PCR allows the user to quantify the nucleic acid template by analysing the amount of DNA produced during each cycle of PCR. The technique relies on the fluorescence of a reporter molecule that increases as product accumulates with each cycle of amplification. In this study, the reporter molecule was a non-specific DNA binding dye, SYBR® Green, which causes an increase in intensity of the fluorescent emissions when it binds the minor Groove of double stranded DNA. As more double stranded amplicons are produced, the SYBR green dye signal will increase. This technique makes quantitation of DNA and RNA easier and more precise than past methods.

Vif has been reported to specifically function in the early HIV reverse transcription in newly infected target cells (Bishop et al., 2004a, Bishop et al., 2004b)(also see Chapter 6). Experiments were performed to investigate whether ZIN decreased virus infectivity by acting at a stage before or after viral reverse transcription in target cells. HuT-78 cells were infected with virus stocks produced in the presence or absence of exogenously expressed ZIN, and newly reverse-transcribed DNA levels in the target HuT-78 cells were measured at 16 hr and 24 hr post-infection (pi) using Real Time PCR.

Equivalent amounts (10 ng of HIV p24) of each of 4 virus stocks produced from 293T cells co-transfected with pNL4.3 and pFlag-ZIN, or pNL4.3 and pFlag, or pNL4.3 (Δ*vif*) and pFlag-ZIN, or pNL4.3 (Δ*vif*) and pFlag (see section **2.2.12**) were used. The pNL4.3 (Δ*vif*) mutant was also used to determine whether the negative

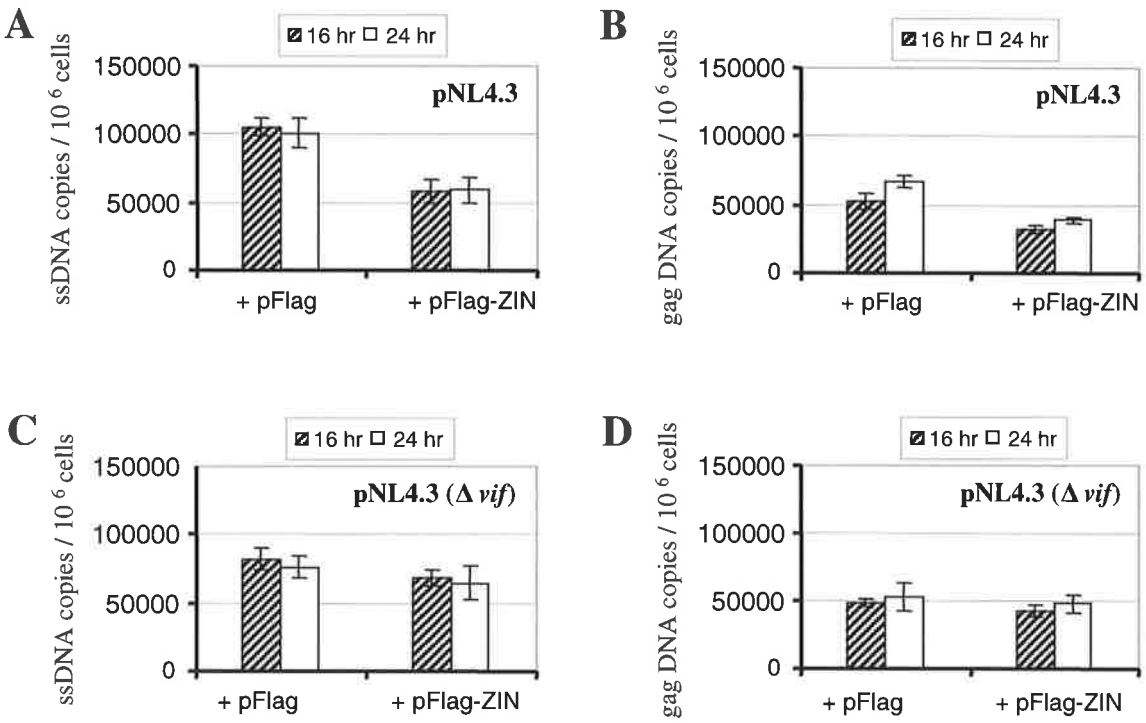
effect was dependent on the specific interaction between ZIN and Vif. Viral reverse transcription was monitored using one primer pair to detect the early strong-stop HIV DNA and another primer pair to detect later viral DNA spanning the U5/Gag region. To avoid secondary infection by progeny virus, cells were harvested for viral DNA analysis within the first 24 hr of infection. At 16 hr pi and 24 hr pi there was approximately 50% less early strong-stop HIV DNA (Figure 4.8 A) in cells infected with HIV wild-type virus produced in the presence of co-transfected pFlag-ZIN, when compared with DNA synthesis in cells infected with HIV produced in the presence of control pFlag (Figure 4.8 B). There was no difference, however, when HIV DNA levels were compared in cells infected with Vif defective HIV NL4.3 (Δ*vif*) produced in the presence or absence of co-expressed ZIN (Figure 4.8 C&D). These results indicate that ZIN expressed in virus producing cells interferes with a step(s) prior to or including HIV reverse transcription in the newly infected target cells. Since this occurred with wild-type but not with Δ*vif* virus, this result suggests that ZIN in virus producing cells mediates its inhibitory effect on progeny virus by some form of interaction with Vif.

4.5.4 Discussion

Infection experiments in HuT-78 cells demonstrated that exogenously expressed ZIN could cause a decrease of in the infectivity of progeny virus relative to p24 level. In contrast, expression of Flag, or two other exogenously expressed proteins GFP and Triad 3au included in preliminary experiment (details not shown), did not have any effect on the viral infectivity/replication, suggesting that the action of ZIN was not a non-specific result of the transfection process, and required the full length functional protein.

Figure 4.8 Virus produced in cells over-expressing ZIN has impaired reverse transcription in newly infected cells.

Levels of HIV-1 strong-stop DNA (A and C) and U5/Gag DNA (B and D) in 10^6 HUT-78 cells infected with 10 ng p24 of HIV virus stocks, 16 hr and 24 hr post infection. Virus stocks were produced from 293T co-transfected with pNL4.3 and pFlag or pFlag-ZIN (A and B), or PNL4.3 (Δvif) and pFlag or pFlag-ZIN (C and D). Viral DNA synthesis was quantified by real-time PCR. DNA copy number values represent the average of four PCR measurements from duplicate infected wells. Error bars represent the standard deviation of triplicate samples.


A
16 hr 24 hr
ssDNA copies / 10 6 cells
150000
100000
50000
0
pNL4.3
+ pFlag
+ pFlag-ZIN
B
16 hr 24 hr
gag DNA copies / 10 6 cells
150000
100000
50000
0
pNL4.3
+ pFlag
+ pFlag-ZIN
C
16 hr 24 hr
ssDNA copies / 10 6 cells
150000
100000
50000
0
pNL4.3 (Δ vif)
+ pFlag
+ pFlag-ZIN
D
16 hr 24 hr
gag DNA copies / 10 6 cells
150000
100000
50000
0
pNL4.3 (Δ vif)
+ pFlag
+ pFlag-ZIN

Real Time PCR analysis indicated that this action of ZIN operated before or at the stage of reverse transcription in infected target cells and required the presence of Vif. Vif has been shown to be important for the efficient reverse transcription of HIV in target cells by affecting the late stages including assembly and packaging in producer cell (Bishop et al., 2004a, Bishop et al., 2004b). HIV-1 completes these late steps in the cytoplasm of the producer cells. Confocal analysis showed ZIN had obvious co-localisation with Vif in the nuclei of 293T cells co-transfected with pMyc-Vif and pFlag-ZIN, while ZIN localised mainly at the cell membrane and cytoplasm in 293T cells co-transfected with pFlag-ZIN and control plasmid pMyc. Nuclear co-localisation of ZIN and Vif may reflect a change in intracellular distribution of this ZIN, leading to more efficient virus replication by means to be clarified

Real Time PCR showed that at 16 hr pi and 24pi there was an approximately 50% decrease in early strong-stop HIV DNA, and gag region DNA levels (Figure 4.8) in cells infected with HIV-1 virus produced in the presence of co-transfected pFlag-ZIN compared with control (pFlag vector alone). These results are clearly consistent with the decrease in viral infectivity measured using the HeLa-CD4-β-gal indicator cells (Figure 4.7). Furthermore the DNA synthesis data indicate that ZIN expressed in virus producing cells affects a step prior to or including reverse transcription. Although the size of the effect was small, it was a consistent finding in three separate experiments (section **4.5.3**), and could result in a significant effect on virus multiplication when amplified over a series of viral replication cycles.

Chapter 5

Vif Site-Directed Mutagenesis

5.1 Introduction

As described in section **2.2.16**, Alexander *et al.* isolated HIV-1 strain AY064706 from a HIV positive daughter and mother who had maintained consistently low plasma levels of human immunodeficiency virus type 1 RNA, as well as normal and stable levels of CD4+ T-cells for at least 15 years (Alexander et al., 2002). To map the determinant of the poor growth of the patients' isolates, the authors performed sequence analysis of the LTR and Vif regions of the isolates and compared them to complete clade B consensus sequences. A two-amino-acid insertion mutation (aspartic acid and serine, DS) in Vif was proposed to be essential for the poor HIV-1 replication. To test this hypothesis, the authors demonstrated that recombinant HIV-1, engineered with Vif sequences from the daughter, replicated in PBMC to levels approximately 20-fold lower than that of wild type. In addition, removal of the insertion mutation from this recombinant restored replication efficiency to wild-type levels, while introduction of the insertion mutation into wild-type Vif sequences resulted in greatly decreased replication. These results suggested that the two-amino-acid (DS) insertion mutation in the Vif gene was essential for the low viral load phenotype. In the same study, Vif protein with a DS insertion mutation displayed an electrophoretic migration pattern of two abundant bands lower than 18 Kd ladder band that were distinct from that of wild-type Vif protein (23 Kd) protein, suggesting an aberrant polypeptide cleavage caused by the mutation. It was proposed that this

mutation might lead to a low viral load by interfering with the ability of Vif protein to inhibit a putative viral inhibitor factor (Alexander et al., 2002).

Since one of the clones isolated from the human leukocyte cDNA library using the yeast- 2- hybrid system 3 (Lake et al., 2003), might represent such an inhibitor, an investigation was done whether the two-amino-acid DS mutation led to suppression of the interaction between Vif and either of the clones. If so, this could provide a mechanism for the lower replication capacity of the virions. This work was performed before the effect of ZIN on HIV-1 replication and infectivity was identified (Chapter 4). This section of the thesis describes the preparation of the DS mutant.

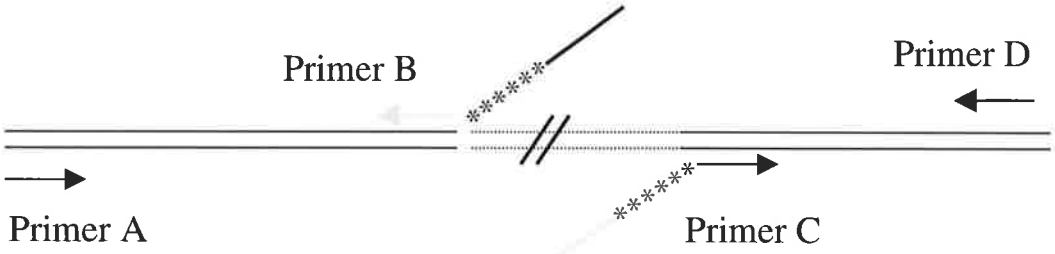
5.2 Experimental principle

To create the Vif DS mutant, the overlap insertion PCR technique was used (Figure 5.1). Two rounds of PCR and four primers (represented by arrows indicating direction of priming) were used. (1) Primer B and Primer C were designed with a long 5' extension, to ensure they: i) bound to the template at either side of the intended splice site; ii) introduced site directed mutations (represented by ******); and iii) were complementary to each other (represented by shading scheme). The first round PCR used the template and primers A and B in one tube and the template and primers C and D in a second tube producing PCR products AB and CD respectively (2). Both PCR products incorporated the desired mutation, and were complementary at one end. The second round PCR required both first round PCR products AB and CD, and the flanking primers A and D. After the first denaturation step, product AB can bind to the complementary region in product CD, and prime DNA synthesis (3).

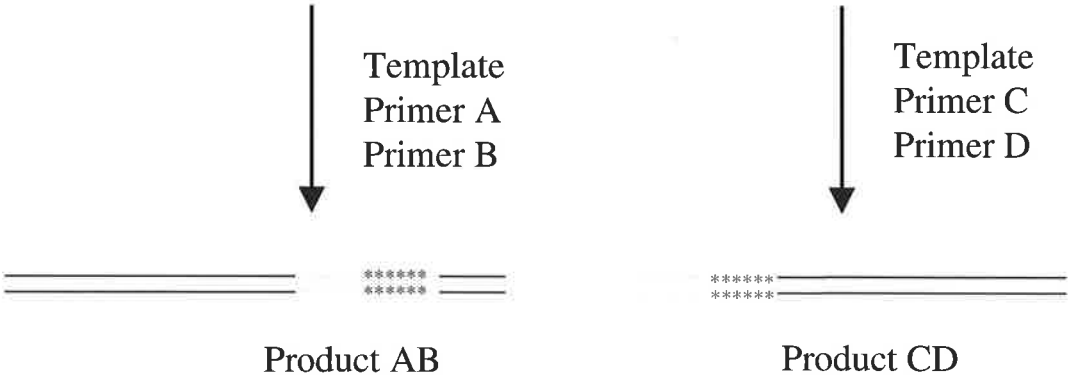
Figure 5.1 Overlap Insertion PCR Technique.

The Vif DS mutant cDNA was constructed by amplifying two cDNA products AB and CD (see Fig.5.2). AB was amplified using primer A, specific for the 5' terminus of Vif sequence containing an introduced *EcoRI* site, and primer B specific for a sequence within the Vif ORF and also encoding a 6 base TACTAT sequence insertion. CD was amplified using primer C, specific for a sequence within the Vif ORF and encoding a 6 base ATAGTA sequence insertion and primer D specific for the 3' terminus of Vif sequence containing an introduced *SalI* site (described in table **2.1**). The second PCR was performed using mixed products AB and CD as templates and primers A and D described above.

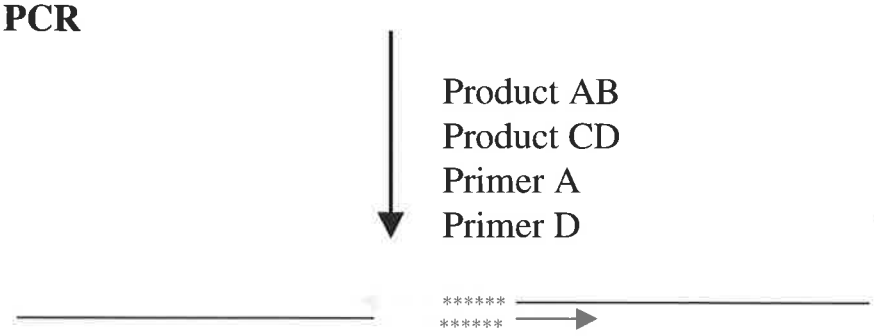
A: Template Priming



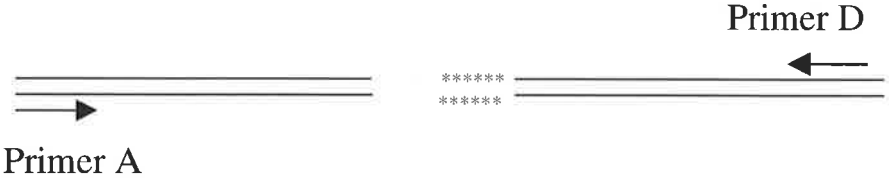
B: 1st round PCR



C: 2nd round PCR
1st cycle



D: 2nd round PCR
subsequent cycles



In subsequent cycles, flanking primers (Primer A and Primer D) were used to prime DNA synthesis (D).

5.3 Result and discussion

As described in section **2.2.16**, using a conventional PCR technique, two PCR products were amplified from the first round, which were around 200 bps and 400 bps respectively (Figure 5.2). In the second round PCR, a fragment of 582 bps was produced (Figure 5.3). Since primers A and D contained *EcoRI* and *SalI* enzyme digestion sites respectively, this product was subjected a double digestion with enzymes *EcoRI* and *SalI* and subcloned into vector pCMV-Myc vector. Sequence analysis showed it was identical to the sequence of NL4.3 vif except for the intended 6- base pair insertion at position 185-190 (following to the NL4.3 vif sequence numbering convention; Figure 5.4). This 6 extra base pair insertion corresponded to the introduction of amino acid insertion at position 62 and 63 but also unavoidably led to a threonine to alanine substitution at amino acid position 64 as described by Alexander *et al.* (Figure 5.5). This substitution in Vif was not shown any importance to the poor replication (Alexander et al., 2002).

We previously reported that NVBP represented a candidate for a Vif related inhibitor of HIV replication because it was present only in non-permissive cells and interacted with Vif in yeast AH109 strain (Lake et al., 2003). However, data in this thesis showed NVBP did not interact significantly with wild type HIV-1 Vif in the human cells tested (discussed in section **3.3**). Therefore, it was decided not to do further research on the interaction between NVBP and the Vif DS mutant. ZIN was shown to interact with HIV-1 Vif in human cells (Chapter 4); however, as it is present in both

Figure 5.2 Vif DS PCR amplification 1

Lane 1: the upstream region of Vif DS (AB) amplified by primers Vif DS A and Vif DS B (listed in table **2.1**)

Lane 2: the downstream region of Vif DS (CD) amplified by primers Vif DS C and Vif DS D (listed in table **2.1**)

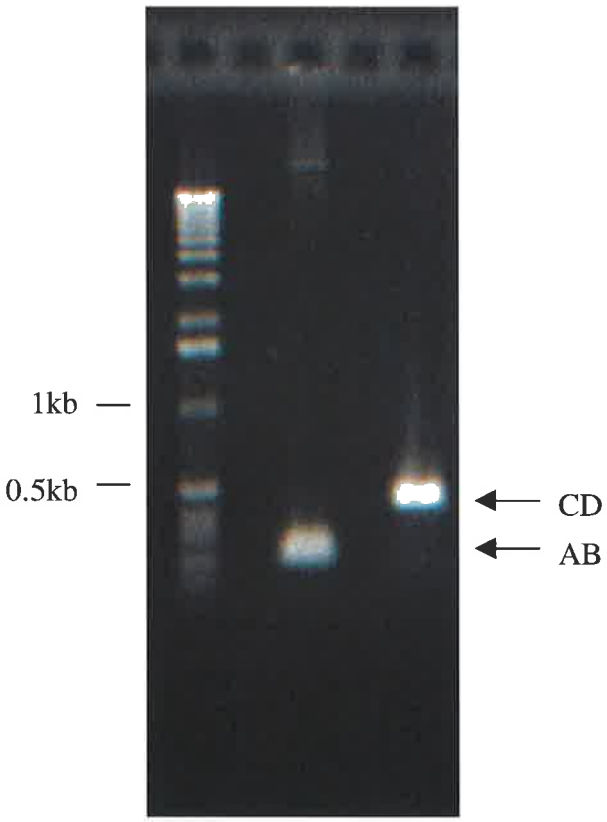

1kb
0.5kb
CD
AB
1
2

Figure 5.3 Vif DS PCR amplification 2

The arrow indicates the Vif DS fragment, amplified by using primers A and D and the products AB and CD as template (Fig.5.2).

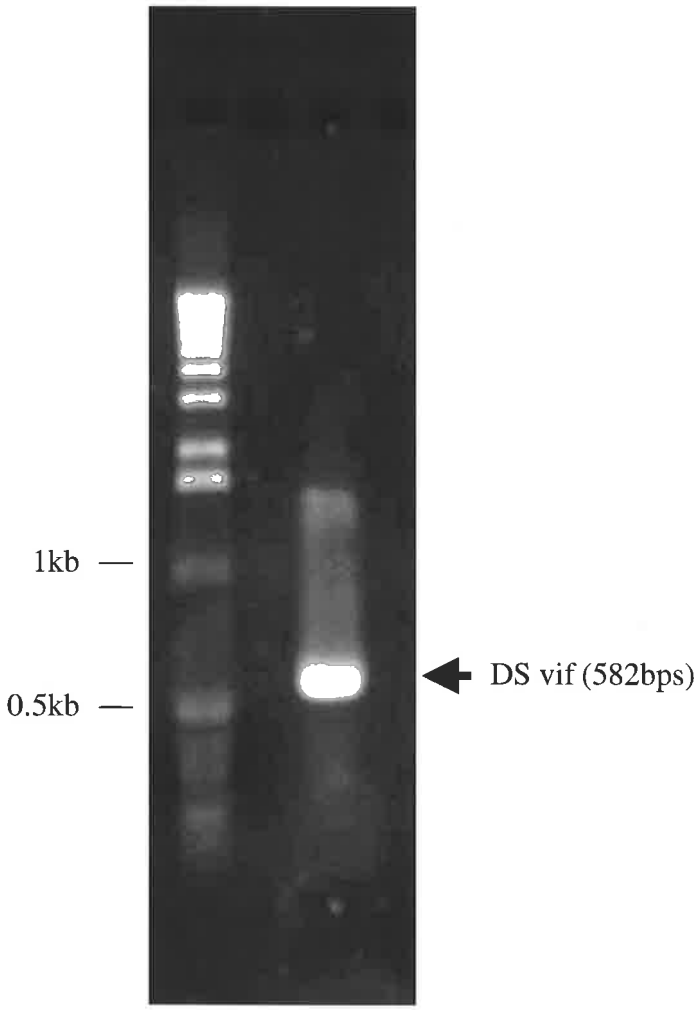

1kb
0.5kb
DS vif (582bps)

Figure 5.4 Nucleotide sequence comparison of Vif DS and NL4.3 Vif

Vif DS sequence analysis was performed using BioEdit program. NL4.3 Vif sequence was adapted from GenBank™. The dots indicate the nucleotides of Vif DS identical to NL4.3 Vif. Six additional nucleotides were inserted from position 185 of NL4.3 Vif. The Vif DS sequence was the same as the clone AY064706 reported by Alexander et al.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
(NL4-3)Vif       ATGGAAAACA GATGGCAGGT GATGATTGTG TGGCAAGTAG ACAGGATGAG
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
(NL4-3)Vif       GATTAACACA TGGAAAAGAT TAGTAAAACA CCATATGTAT ATTTCAAGGA
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
(NL4-3)Vif       AAGCTAAGGA CTGGTTTTAT AGACATCACT ATGAAAGTAC TAATCCAAAA
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
(NL4-3)Vif       ATAAGTTCAG AAGTACACAT CCCACTAGGG GATG------ CTAAATTAGT
DS vif           .......... .......... .......... ....ATAGTA ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         210        220        230        240        250
(NL4-3)Vif       AATAACAACA TATTGGGGTC TGCATACAGG AGAAAGAGAC TGGCATTTGG
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         260        270        280        290        300
(NL4-3)Vif       GTCAGGGAGT CTCCATAGAA TGGAGGAAAA AGAGATATAG CACACAAGTA
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350
(NL4-3)Vif       GACCCTGACC TAGCAGACCA ACTAATTCAT CTGCACTATT TTGATTGTTT
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         360        370        380        390        400
(NL4-3)Vif       TTCAGAATCT GCTATAAGAA ATACCATATT AGGACGTATA GTTAGTCCTA
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         410        420        430        440        450
(NL4-3)Vif       GGTGTGAATA TCAAGCAGGA CATAACAAGG TAGGATCTCT ACAGTACTTG
DS vif           .......... .......... .......... .......... ..........
```

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         460        470        480        490        500
(NL4-3)Vif       GCACTAGCAG CATTAATAAA ACCAAAACAG ATAAAGCCAC CTTTGCCTAG
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         510        520        530        540        550
(NL4-3)Vif       TGTTAGGAAA CTGACAGAGG ACAGATGGAA CAAGCCCCAG AAGACCAAGG
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|
                         560        570        580
(NL4-3)Vif       GCCACAGAGG GAGCCATACA ATGAATGGAC ACTAG
DS vif           .......... .......... .......... .....
```

Figure 5.5 Deduced amino acid sequence alignment of Vif DS and NL4.3 Vif

Two additional amino acids DS were confirmed to be inserted from position 62 of NL4.3 Vif amino acid. The six base pair insertion interfered with the original translation frame leading to an adjacent amino acid Threonine (T) to Alanine (A) substitution which was not shown any importance to poor replication of HIV in Alexander's study. Dots showed the amino acids of Vif DS that were identical to NL4.3 Vif.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
VIF OF NL4       MENRWQVMIV WQVDRMRINT WKRLVKHHMY ISRKAKDWFY RHHYESTNPK
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
VIF OF NL4       ISSEVHIPLG D--AKLVITT YWGLHTGERD WHLGQGVSIE WRKKRYSTQV
DS vif           .......... .DST...... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
VIF OF NL4       DPDLADQLIH LHYFDCFSES AIRNTILGRI VSPRCEYQAG HNKVGSLQYL
DS vif           .......... .......... .......... .......... ..........

                 ....|....| ....|....| ....|....| ....|....| ....
                        160        170        180        190
VIF OF NL4       ALAALIKPKQ IKPPLPSVRK LTEDRWNKPQ KTKGHRGSHT MNGH
DS vif           .......... .......... .......... .......... ....
```

non-permissive and permissive cells (Chen et al., 2002), it is unlikely to be the major factor governing non-permissiveness. Later, Real Time PCR analysis showed that exogenously expressed ZIN decreased the production of HIV-1 RT product by only approximately 50% in the presence of Vif; in contrast, the Vif DS HIV-1 mutant was reported to replicate in PBMC to levels approximately 20-fold lower than wild-type virion (Alexander et al., 2002). This suggested the association between ZIN and Vif (or DS mutant) is not sufficient to fully explain the mechanism of Vif function, and therefore further study on ZIN and the Vif DS mutant was not performed. However, more recent research showed that Vif is needed to overcome a cellular inhibitor (CEM15/APOBEC3G) present in non-permissive cells (discussed in Chapter 6). It would be interesting to examine the relation of CEM15/APOBEC3G to this Vif DS mutant. Vif protein cleavage resulting from the DS insertion may damage its activity to overcome CEM15/APOBEC3G. The DS mutant prepared above would be available for further research in this area, but because of time constraint this was not pursed in this study.

Chapter 6

General discussion and future work

6.1 Discussion

The *vif* gene of human immunodeficiency virus type 1 (HIV-1) is highly conserved among lentiviruses (Oberste & Gonda, 1992) and encodes a highly basic 23 kDa protein. Using fractionation of acutely infected T cells or transiently transfected HeLa cells Karczewski *et al.* demonstrated the presence of Vif in a soluble and a cytoskeletal form, and to a lesser extent in a detergent-extractable form (Karczewski & Strebel, 1996). Indirect immunofluorescence staining demonstrated Vif in a diffuse cytoplasmic distribution mostly not in association with the Golgi complex (Guy et al., 1991). Subcellular fractionation of transfected COS cells and HIV-1-infected Jurkat and CEM cells demonstrated that Vif existed in the cytoplasm in both a soluble cytosolic form and membrane-associated form (Goncalves et al., 1994). In contrast, immunofluorescence staining of cells infected with FIV-34TF10 and fixed with formaldehyde showed that Vif protein was typically localised in the nucleus (Chatterji et al., 2000). Consistent with Goncalves et al's result, in this study, confocal microscopy of 293T cells transfected with plasmid Myc-Vif indicated Vif was distributed in both the cytoplasm and nuclei in the absence of overexpressed ZIN.

Vif protein is not essential for HIV-1 replication in permissive cells, such as Hela-CD4, 293T cells, or in semipermissive cells such as SupT1 (Gabuzda et al., 1992, Sakai et al., 1993b). However, Vif is required for productive infection of non-

permissive cells that are natural targets for infection *in vivo*, including CD4-positive T lymphocytes and macrophages, and the H9 T-lymphoblastoid cell line (Blanc et al., 1993, Gabuzda et al., 1992, von Schwedler et al., 1993). This cell specificity in Vif requirement led to proposals that (1) permissive cells may contain a cellular protein that functions like Vif, thereby enabling active replication of vif deficient HIV-1 (Trono, 1995, Volsky et al., 1995) or alternatively, (2) nonpermissive cells may contain an inhibitor of HIV-1 replication that is counteracted by Vif. To test these distinct hypotheses, Simon *et al* and Madani *et al* fused permissive with non-permissive cells and demonstrated that the restrictive phenotype of non-permissive cells was dominant over the permissive phenotype (Simon et al., 1998a; Madani & Kabat, 1998). These findings suggested that nonpermissive cells contain an activity inhibiting HIV-1 replication which is counteracted by Vif.

The most likely candidate for such an inhibitor of HIV-1 replication in non-permissive cells is the recently described APOBEC3G, also known as CEM 15 (Sheehy et al., 2002). APOBEC3G is a cytidine deaminase, which induces C to U changes in viral minus strand DNA during reverse transcription. Vif defective virus produced in the presence of APOBEC3G was found to be non-infectious or reduced in infectivity in non-permissive target cells, depending on the varying doses of pAPOBEC3G co-transfected together with either wild-type or *Δvif* provirus expression plasmids within producer cells. The major defect identified was a high frequency of G to A substitution in the viral DNA synthesised in newly infected target cells (Harris et al., 2003, Lecossier et al., 2003, Mangeat et al., 2003, Sheehy et al., 2002, Zhang et al., 2003). Klarmann showed that a high frequency of nucleotide changes might make the viral DNA susceptible to excision by host DNA-

repair enzymes, thereby impairing plus-strand DNA synthesis (Klarmann et al., 2003). Vif acts to suppress this inhibitory effect of APOBEC3G in non-permissive cells, by inducing degradation of APOBEC3G (Lecossier et al., 2003, Mangeat et al., 2003, Sheehy et al., 2003) and by preventing it from being packaged into virions (Mariani et al., 2003, Sheehy et al., 2002, Zhang et al., 2003). Vif has also been shown to interact with cellular proteins Cul5, Elongins B and C, and Rbx1 to form a Skp1-Cullin-F-box (SCF)—like complex. The ability of Vif to suppress the antiviral activity of APOBEC3G was specifically dependent on Cul5-SCF function, as shown by the fact that a Vif mutant that interacted with APOBEC3G but not with Cul5-SCF was functionally inactive. Thus, it is believed that Vif interacts both with APOBEC3G and Cul5-SCF to induce ubiquitination and degradation of APOBEC3G (Yu et al., 2003). In addition to binding APOBEC3G, Vif has been associated with a number of other activities, such as binding viral RNA (Zhang et al., 2000a) and NC (Huvent, I. *et al* 1998), and has also been shown to affect the stability of the viral nucleoprotein core (Hoglund et al., 1994, Ohagen & Gabuzda, 2000, Simon & Malim, 1996). Despite earlier controversial reports, recent evidence suggests that Vif is incorporated into virions (Kao et al., 2003a, Khan et al., 2001, Liu et al., 1995a). These properties of Vif suggest it may have other functions independent of APOBEC3G. Indeed a number of cellular factors have been shown to interact with Vif in mammalian cells. These include tyrosine kinase HcK (Hassaine et al., 2001), an ATP-binding protein HP68 (Zimmerman et al., 2002), and lymphocyte-specific nuclear body protein Sp140 (Madani et al., 2002).

Prior to the work of this thesis, eight clones from a human leucocyte cDNA library had been shown to interact with Vif in the yeast 2 hybrid system 3 and had been

isolated (Lake et al., 2003). In the present study, the interactions between Vif and two of these clones - a partial clone of Triad 3 named Triad 3au, and NVBP were examined in more detail. In co-immunoprecipitation experiments, Triad 3au was shown to interact with HIV-1 Vif in co-transfected 293T cells; in contrast, the possible interaction between HIV-1 Vif and NVBP suggested from the yeast -2-hybrid experiments was found not to occur in co-transfected 293T cells. Confocal microscopy of co-transfected 293T cells demonstrated that co-localisation of Triad 3au and Vif occurred mainly in the cytoplasm and cellular membrane and co-localisation could not be detected in cell nuclei. Triad 3au mRNA was detected in both permissive and non-permissive human cell types by Northern blot, as 3 kb and 6 kb transcripts similar in size to that described for ZIN specific RNA by Chen *et al* (Chen et al., 2002). However, whether the 488 amino acid, ZIN protein is encoded from the 3 kb or 6 kb transcripts is unclear, since both RNA species contain the entire ZIN open reading frame. The presence of Triad 3au specific RNA in both non-permissive and permissive cells would also suggest that ZIN alone is not a determinant of non-permissiveness as has been suggested for APOBEC3G.

The amino acid sequence deduced from the Triad 3au cDNA clone nucleotide sequence was part of a hypothetical protein containing novel ring-finger motifs, called Triad 3 (van der Reijden et al., 1999). Triad 3au was found to share exact homology with the central domain of Triad 3 containing four ring-like domains, and with more recently identified functional protein, zinc finger protein inhibiting NF-*k*B, or ZIN (GenBank™ accession number NP_061884). As described in section **4.1**, in TNF-α stimulated human embryonic kidney 293 cells that are over-expressing ZIN, ZIN inhibits NF-*K*B activation by interfering with the induced signal

transduction pathway through its interaction with the TNF-α receptor adaptor protein, RIP. This interaction is also associated with RIP- and TNF-induced apoptosis in these same cells (Chen et al., 2002).

To further characterise ZIN activity, total RNA was extracted from the A3.01 cells, and ZIN cDNA was synthesized by RT PCR using ZIN cDNA specific 5' and 3' terminal primers based on published sequence (Chen et al., 2002), and was cloned into the protein expression plasmid pCMV-3Flag. ZIN protein was expressed in transfected 293T cells, and a GST-Vif fusion protein was produced in *E.coli* BL21 cells. ZIN was then shown to interact with purified Vif protein *in vitro* using a pull-down assay, suggesting a direct interaction between ZIN and Vif. Consistent with this result, co-immunoprecipitation experiments performed on lysates of 293T cells co-transfected with pMyc-Vif and pFlag-ZIN indicated a clear interaction between Vif and ZIN in these cells.

The finding that both ZIN and the shortened form of the protein Triad 3au interacted with Vif suggest that the central portion of ZIN, encoded by Triad 3au and containing all 4 RLDs, contain motifs involved in Vif binding. In transfected cells expressing ZIN alone, ZIN localised mainly in cytoplasm (Figure 4.5 A), consistent with the previous report by Chen *et al.* that natural ZIN expression occurred in cytoplasm. Meanwhile, Vif protein was detected in both cytoplasm and nuclei of in transfected cells expressing Vif alone in this study and other studies (Figure 3.12 A, B) (Chatterji et al., 2000, Goncalves et al., 1994, Goncalves et al., 1995, Simon et al., 1997). In co-transfected cells expressing ZIN and Vif, ZIN became co-localized with Vif in cell nuclei. This suggests that the interaction between ZIN and Vif resulted in

a relocalisation of ZIN, which may be involved in a biological effect on viral infectivity or replication of HIV-1 during infection.

To determine if the interaction between Vif and ZIN had any effect on HIV-1 replication, the infectivity of virus produced from cells with and without exogenous ZIN was studied, relative to the concentration of p24 protein as a marker of total virion production. This study demonstrated that virus replication, as indicated by p24 levels in infected cell culture supernatants, proceeded at a similar rate whether or not exogenous ZIN was expressed in the producer cells. ZIN is a component of the TNF receptor mediated signal transduction pathway involved in inhibiting NF-*k*B activity in TNFα stimulated cells. Importantly NF-*k*B is critical to many aspects of cellular transcription, including transcription from the HIV-1 LTR promoter. Despite this, the above result indicated that HIV-1 transcription proceeded normally in cells expressing exogenous ZIN, hence suggesting NF-*K*B activity was not affected by ZIN overexpression. In contrast, virus produced from cells expressing exogenous ZIN showed a lower $TCID_{50}$ than virus produced without ZIN. To examine this further, virus DNA replication in target cells was also analysed.

Firstly, using HeLa-CD4-LTR-β-gal as reporter cells, exogenous ZIN expression in virus producer cells was shown to reduce the infectivity of wild type virus by a factor of 2 but not the infectivity of Vif defective virus, indicating that this effect of ZIN was dependent on the presence of Vif. Moreover, since activation of HeLa-CD4-LTR-β-gal reporter cells depends on successful virus entry and completion of reverse transcription and integration by infecting virus, this finding suggested that the reduction in infectivity of wild type virus produced in ZIN expressing cells occurred

during these early steps of HIV replication. Next, it was shown that levels of newly reverse transcribed viral DNA were reduced 2-fold in HuT-78 target cells infected with virus produced in cells expressing co-transfected ZIN, compared with the virus produced in the absence of ZIN. Again, this reduction was shown to depend on Vif expression in virus producer cells. Since the producer cells 293T were demonstrated not to contain APOBEC3G using Northern blotting (Sheehy et al., 2002), this reduction in virus infectivity is unlikely to be related to the inhibitor APOBEC3G. In contrast, exogenous expression of Triad 3au in virus producer cells had no effect on viral infectivity (discussed in section **4.5.4**). This would suggest that interaction between Vif and Triad 3au or the central portion of ZIN alone is not sufficient to affect virus infectivity, and that other functional domains of ZIN present in the N-terminal and or C-terminal proline-rich domains are required. For example, proline rich domains have been shown to be important for the interaction with several cell proteins (see section **4.4**). In the current study, in the presence of Vif the full length ZIN protein showed a different cellular localisation (mainly in cell nuclei, see Figure 4.5 I) to that of Triad 3au (only in cytoplasm or cell membrane, see Figure 3.15 D) possibly due to presence of the extra structure N-terminal and C-terminal domains contained in the full ZIN protein. This may be a factor contributing the functional difference between Triad 3au and ZIN (see section **4.4**).

Taken together, our results show that an interaction between ZIN and Vif in virus producer cells leads to reduced HIV replication in newly infected target cells, and this interference can not be interpreted by the known mechanism involving APOBEC3G inhibition of viral DNA synthesis. Therefore the inhibitory effect that ZIN mediates through its interaction with Vif implies that Vif has additional roles on

HIV infectivity. Such functions of Vif may act in the late stages of viral replication in the virus producer cells, but be manifested in the early steps of viral replication in target cells.

Vif has been shown to have other possible roles. Vif is synthesised in the late stage of HIV-1 replication by a process that is dependent on the Rev regulatory protein (Garrett et al., 1991, Schwartz et al., 1991). Vif has been shown to interact with viral RNA and NC proteins at this late stage of replication during virus assembly, and also to be incorporated into virions (Khan et al., 2001, Ohagen & Gabuzda, 2000). Dettenhofer *et al* demonstrated that Vif enhanced the annealing of $tRNA3^{Lys}$ to viral genomic RNA, a necessary step for efficient initiation of reverse transcription (Dettenhofer et al., 2000). These workers also suggested that the initial point of contact between Vif and genomic RNA might take place in the nucleus where Vif has been shown to localize (Chatterji et al., 2000, Goncalves et al., 1995, Simon et al., 1997) before becoming associated with Gag assembly complexes in the cytoplasm. It has also been reported that deletion from Vif of the SLQYL motif, which is identical to a motif in the double-stranded RNA binding protein XlrbpA from X.*laevis*, resulted in severe impairment or complete abolition of Vif function (Dettenhofer et al., 2000, Khan et al., 2002). Zhang *et al.* demonstrated that Vif mutants that lose the RNA binding activity *in vitro* did not support vif-deficient HIV-1 replication in H9 T cells, suggesting that the RNA binding capacity of Vif is important for its function (Zhang et al., 2000a). Thus, Vif has other potential biologically relevant functions including its interaction with genomic RNA, its role in primer tRNA binding, its interaction with viral Gag proteins during virus assembly, and its role in maintaining the stability of the virus core (Ohagen &

Gabuzda, 2000). One or more of these functions might be affected by the interaction with ZIN.

In addition, Vif has been shown to function within virions to regulate virus infectivity. In virions Vif is proteolytically cleaved and this process was shown to be important for virus infectivity, but only at proper levels of Vif expression (Akari et al., 2004, Khan et al., 2002). Akari *et al* found that when Vif was expressed at low levels in non-permissive H9 cells, it increased virus infectivity in a dose-dependent manner. Surprisingly, however, the range of Vif required for optimal virus infectivity was narrow, and further increases in Vif severely reduced viral infectivity. Inhibition of viral infectivity at higher levels of Vif was cell type-independent and was associated with an accumulation of Gag-processing intermediates (Akari et al., 2004). More recently Vif has also been found in reverse transcription complexes in infected cells (Carr *et al*, submitted). Thus, overexpression of ZIN in these experimental settings may compete and interfere with any of the above functions of Vif, which would be consistent with the reduced reverse transcription observed following HIV infection. The effects of ZIN on Vif function may therefore occur during virus assembly, maturation or egress, eg by binding Vif and blocking Vif from associating with NC or RNA, leading to production of virus with reduced infectivity. Alternatively, ZIN may be packaged and either inhibit Vif function within the virion or modify the levels of Vif packaged in virions.

Interestingly, apart from the well-characterised function of ZIN in regulating NF*K*B activity in TNF-α stimulated cells (Chen et al., 2002), ZIN has also been shown to share sequence homology with E3 ubiquitin ligases (eg. AY177398). Several

proteins with the TRIAD domain have E3 ligase activity, including Parkin, the double ring finger protein (Dorfin), and the human homologue of drosophila ariadne (ARIH1, also known as HHARI) (Chung et al., 2001, Moynihan et al., 1999, Shimura et al., 2001, Zhang et al., 2000b). Thus, it is possible that ZIN might function as an E3 ligase. Ubiquitination is a posttranslational protein modification that requires ATP and three different proteins, ubiquitin activation enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3). Free ubiquitin is recruited to E1 and transferred to E2. E2 ubiquitin conjugases bind to Ring finger domains on a multitude of E3 ligases. E3 ligases specify protein targets by recruiting cellular proteins, through specific adapter domains, and by recruiting E2 conjugases that direct their ubiquitination. Polyubiquitinated target proteins are recognized by the 26S proteasome, which unfolds and degrades the targeted protein (Ben-Neriah, 2002, Geimonen et al., 2003, Haas & Siepmann, 1997). Alternatively monoubiquitination of target proteins often confers a regulatory or functional role in the target proteins. ZIN therefore may act as an ubiquitin-protein ligase or as part of a complex that may be involved in regulating Vif function or cellular turnover. Recently, Chuang and Ulevitch have identified a cellular protein, termed "Triad 3A", that is identical to ZIN except for a 378 amino acids N-terminal sequence extension. Triad 3A was shown to possess an E3 ubiquitin-protein ligase activity, and enhanced ubiquitination and proteolytic degradation of selected Toll-like receptor target proteins (Chuang & Ulevitch, 2004). Whether ZIN also possesses E3 ubiquitin activity and involves in Vif ubiquitination and degradation is currently under investigation in our laboratory.

In conclusion, this work has identified and characterised an interaction between HIV-1 Vif and a cellular protein, ZIN. Furthermore it has been demonstrated that overexpression of ZIN in HIV producer cells results in production of virus with reduced infectivity. In contrast to APOBEC3G, an inhibitor of HIV replication expressed exclusively in non-permissive cells, ZIN suppresses virus infectivity in a Vif dependent manner and is expressed in both non-permissive cell and permissive cell types. A greater understanding of normal ZIN function and its interaction with Vif will aid in uncovering further details of Vif function.

6.2 Future work

HIV-1 Vif is required during the last stages of virus production for the suppression of an innate antiviral activity of CEM15/APOBEC3G in human T lymphocytes. In this study, the cellular factor ZIN has also been shown to interact with HIV-1 Vif, and overexpression of ZIN suppressed the infectivity of newly synthesised virus. Single-cycle viral infectivity assay showed that the effect of ZIN on viral infectivity was dependent on the interaction between Vif and ZIN (section **4.5.2.**). However, the exact molecular mechanism of this effect remains to be elucidated. HIV-1 virions contain 10-100 molecules of Vif per virion (Camaur & Trono, 1996, Fouchier et al., 1996, Liu et al., 1995b). Whether ZIN influences Vif incorporation or whether ZIN itself is packaged into virions by virtue of its interaction with Vif is unclear. Future studies should determine whether levels of Vif in the virions are affected by ZIN expression in the producer cell, and whether ZIN is present in virions. To determine the extent of virion incorporation of Vif and ZIN, wild type and Δvif virions could be produced in 293T cells in the presence or absence of overexpressed ZIN. Purified virus would then be analysed by western blot for Vif and ZIN protein, and

normalized for viral protein by p24 protein detection. This result would clarify whether ZIN is incorporated in virions and whether such incorporation requires functional Vif. This would help clarify whether ZIN functions in the virus producer cell, within the virion, or within newly infected cells.

A larger cellular protein Triad 3A that contains the complete ZIN sequence was recently reported to act as an E3 ubiquitin ligase (discussed above), ZIN (in frame) shares 99% nucleotide sequence homology to Triad 3A suggesting that ZIN may share this function. To examine whether ZIN has E3 ubiquitin ligase function involved in Vif ubiquitination, further experiments should be performed using ubiquitin specific antibody. Initially, experiments could be done singly transfecting ZIN or co-expressing ZIN with a plasmid expressing HA-tagged ubiquitin to determine whether ZIN autoubiquitinates using ubiquitin or HA specific antibody for western blotting as autoubiquitination is a common feature of E3 ubiquitin ligases. Additionally, Myc-tagged Vif could be expressed together with or without HA-ubiquitin in the presence or absence of Flag-tagged ZIN, and the extent of ZIN-dependent Vif ubiquitination could indicate whether ZIN enhances ubiquitination of Vif. This could be tested using a co-immunoprecipitation approach similar to that described in this thesis. Since endogenous ZIN may also be involved in Vif ubiqutination, the extent of Vif ubiquitination with or without overexpressed ZIN should be compared. If Vif is ubiqutinated in the presence of overexpressed ZIN to a greater extent than that seen without overexpressed ZIN, this result could suggest ZIN may have the function of E3 ubiquitination ligase.

Finally, since the data in this study were mostly based on protein overexpression, the physiological role of ZIN needs to be elucidated by experiments involving the endogenous ZIN protein, with or without gene knockout studies. For this purpose, a ZIN specific antibody has been raised in rabbits. Future work should investigate firstly whether endogenous ZIN interacts with Vif during HIV-1 infection using the approach taken in this thesis. Secondly, U937 cells were found to be naturally depleted in ZIN in our laboratory, but this result has not been confirmed. Other possible cells which are naturally depleted in ZIN have not been reported. Therefore, to investigate the functions of endogenous ZIN, a siRNA approach should be developed to knock out endogenous ZIN and investigate the effect on HIV protein expression and virion production. This work will be valuable in defining the extent to which interaction between ZIN and Vif plays a nature role in HIV infection.

This study clearly demonstrated an interaction between HIV-1 Vif and ZIN. The consequence of the interaction between Vif and ZIN when over-expressed in producer cells, was a defect in the infectivity of progeny virus. These virus particles showed reduced reverse transcription in target cells, indicating a defect at some stage of replication prior to or including this step. This effect is established in the producer cells, and may involve viral genome packaging or virus core stability and may or may not be related to the normal action of Vif. Whatever the mechanism of ZIN mediated effects on virus infectivity, this may provide a suitable target for future drug development. For example, a drug could be designed to upregulate endogenous ZIN expression. Endogenous ZIN protein might directly decrease viral infectivity by its interaction with Vif, in the same way that overexpressed ZIN does. In further work, studies to determine the exact binding sites between ZIN and Vif could allow a

ZIN-like protein analogue to be designed, which when delivered to infected cells might affect Vif function in infected cells. These may be particularly relevant in non-permissive cell types including primary T lymphocytes and macrophages, the natural targets of HIV-1 infection *in vivo*. Neutralisation of Vif by a upregulated ZIN or ZIN-like analogue might prevent Vif's interaction with APOBEC3G, a natural cellular inhibitor of HIV-1 infection expressed in these cells, and hence boost the natural innate immunity of the host to HIV-1 infection.

Further knowledge about the interaction between the above molecules and their regulatory effect on HIV replication is very likely to improve our range of therapeutic interventions against HIV infection.

Reference

Akari, H., Fujita, M., Kao, S., Khan, M. A., Shehu-Xhilaga, M., Adachi, A. & Strebel, K. (2004). High level expression of human immunodeficiency virus type-1 Vif inhibits viral infectivity by modulating proteolytic processing of the Gag precursor at the p2/nucleocapsid processing site. *The Journal of Biological Chemistry* **279**, 12355-12362.

Aldrovandi, G. M. & Zack, J. A. (1996). Replication and pathogenicity of human immunodeficiency virus type 1 accessory gene mutants in SCID-hu mice. *Journal of Virology* **70**, 1505-1511.

Alexander, L., Aquino-DeJesus, M. J., Chan, M. & Andiman, W. A. (2002). Inhibition of human immunodeficiency virus type 1 (HIV-1) replication by a two-amino-acid insertion in HIV-1 Vif from a nonprogressing mother and child. *Journal of Virology* **76**, 10533-10539.

Alimonti, J. B., Ball, T. B. & Fowke, K. R. (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *Journal of General Virology* **84**, 1649-1661.

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. & Berger, E. A. (1996). CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272**, 1955-1958.

Amara, R. R., Villinger, F., Altman, J. D., Lydy, S. L., O'Neil, S. P., Staprans, S. I., Montefiori, D. C., Xu, Y., Herndon, J. G., Wyatt, L. S., Candido, M. A., Kozyr, N. L., Earl, P. L., Smith, J. M., Ma, H. L., Grimm, B. D., Hulsey, M. L., Miller, J., McClure, H. M., McNicholl, J. M., Moss, B. &

Robinson, H. L. (2001). Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. *Science* **292**, 69-74.

Apolloni, A., Hooker, C. W., Mak, J. & Harrich, D. (2003). Human immunodeficiency virus type 1 protease regulation of tat activity is essential for efficient reverse transcription and replication. *Journal of Virology* **77**, 9912-9921.

Ball, R. A. & Kinchelow, T. (2003). Injection site reactions with the HIV-1 fusion inhibitor enfuvirtide. *Journal of the American Academy of Dermatology* **49**, 826-831.

Baraz, L., Friedler, A., Blumenzweig, I., Nussinuv, O., Chen, N., Steinitz, M., Gilon, C. & Kotler, M. (1998). Human immunodeficiency virus type 1 Vif-derived peptides inhibit the viral protease and arrest virus production. *FEBS Letters* **441**, 419-426.

Barouch, D. H., Santra, S., Schmitz, J. E., Kuroda, M. J., Fu, T. M., Wagner, W., Bilska, M., Craiu, A., Zheng, X. X., Krivulka, G. R., Beaudry, K., Lifton, M. A., Nickerson, C. E., Trigona, W. L., Punt, K., Freed, D. C., Guan, L., Dubey, S., Casimiro, D., Simon, A., Davies, M. E., Chastain, M., Strom, T. B., Gelman, R. S., Montefiori, D. C., Lewis, M. G., Emini, E. A., Shiver, J. W. & Letvin, N. L. (2000). Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. *Science* **290**, 486-492.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret , S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983). Isolation of a T-lymphotropic

retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**, 868-871.

Baur, A. S., Sawai, E. T., Dazin, P., Fantl, W. J., Cheng-Mayer, C. & Peterlin, B. M. (1994). HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. *Immunity* **1**, 373-384.

Belyakov, I. M., Hel, Z., Kelsall, B., Kuznetsov, V. A., Ahlers, J. D., Nacsa, J., Watkins, D. I., Allen, T. M., Sette, A., Altman, J., Woodward, R., Markham, P. D., Clements, J. D., Franchini, G., Strober, W. & Berzofsky, J. A. (2001). Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nature Medicine* **7**, 1320-1326.

Ben-Neriah, Y. (2002). Regulatory functions of ubiquitination in the immune system. *Nature Immunology* **3**, 20-26.

Benson, R. E., Sanfridson, A., Ottinger, J. S., Doyle, C. & Cullen, B. R. (1993). Downregulation of cell-surface CD4 expression by simian immunodeficiency virus Nef prevents viral super infection. *Journal of Experimental Medicine* **177**, 1561-1566.

Bernstein, H. B., Tucker, S. P., Kar, S. R., McPherson, S. A., McPherson, D. T., Dubay, J. W., Lebowitz, J., Compans, R. W. & Hunter, E. (1995). Oligomerization of the hydrophobic heptad repeat of gp41. *Journal of Virology* **69**, 2745-2750.

Bishop, K. N., Holmes, R. K., Sheehy, A. M., Davidson, N. O., Cho, S. J. & Malim, M. H. (2004a). Cytidine deamination of retroviral DNA by diverse APOBEC proteins. *Current Biology* **14**, 1392-1396.

Bishop, K. N., Holmes, R. K., Sheehy, A. M. & Malim, M. H. (2004b). APOBEC-mediated editing of viral RNA. *Science* **305**, 645.

Blanc, D., Patience, C., Schulz, T. F., Weiss, R. & Spire, B. (1993). Transcomplementation of VIF- HIV-1 mutants in CEM cells suggests that VIF affects late steps of the viral life cycle. *Virology* **193**, 186-192.

Blumenzweig, I., Baraz, L., Friedler, A., Danielson, U. H., Gilon, C., Steinitz, M. & Kotler, M. (2002). HIV-1 Vif-derived peptide inhibits drug-resistant HIV proteases. *Biochemical & Biophysical Research Communications* **292**, 832-840.

Bour, S. & Strebel, K. (2003). The HIV-1 Vpu protein: a multifunctional enhancer of viral particle release. *Microbes & Infection* **5**, 1029-1039.

Bouyac, M., Courcoul, M., Bertoia, G., Baudat, Y., Gabuzda, D., Blanc, D., Chazal, N., Boulanger, P., Sire, J., Vigne, R. & Spire, B. (1997). Human immunodeficiency virus type 1 Vif protein binds to the Pr55Gag precursor. *Journal of Virology* **71**, 9358-9365.

Broussard, S. R., Staprans, S. I., White, R., Whitehead, E. M., Feinberg, M. B. & Allan, J. S. (2001). Simian immunodeficiency virus replicates to high levels in naturally infected African green monkeys without inducing immunologic or neurologic disease. *Journal of Virology* **75**, 2262-2275.

Bryant, M. & Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 523-527.

Bukrinskaya, A., Brichacek, B., Mann, A. & Stevenson, M. (1998). Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse

transcription complex involves the cytoskeleton. *Journal of Experimental Medicine* **188**, 2113-2125.

Burton, A. (2003). Enfuvirtide approved for defusing HIV. *The Lancet Infectious Diseases* **3**, 260.

Callahan, M. K., Popernack, P. M., Tsutsui, S., Truong, L., Schlegel, R. A. & Henderson, A. J. (2003). Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. *The Journal of Immunology* **170**, 4840-4845.

Camaur, D. & Trono, D. (1996). Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *Journal of Virology* **70**, 6106-6111.

Cao, Y., Qin, L., Zhang, L., Safrit, J. & Ho, D. D. (1995). Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. *The New England Journal of Medicine* **332**, 201-208.

Cavert, W., Notermans, D. W., Staskus, K., Wietgrefe, S. W., Zupancic, M., Gebhard, K., Henry, K., Zhang, Z. Q., Mills, R., McDade, H., Schuwirth, C. M., Goudsmit, J., Danner, S. A. & Haase, A. T. (1997). Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. *Science* **276**, 960-964.

Chakrabarti, L. A., Lewin, S. R., Zhang, L., Gettie, A., Luckay, A., Martin, L. N., Skulsky, E., Ho, D. D., Cheng-Mayer, C. & Marx, P. A. (2000). Normal T-cell turnover in sooty mangabeys harboring active simian immunodeficiency virus infection. *Journal of Virology* **74**, 1209-1223.

Chang, F., Re, F., Sebastian, S., Sazer, S. & Luban, J. (2004). HIV-1 Vpr induces defects in mitosis, cytokinesis, nuclear structure, and centrosomes. *Molecular Biology of the Cell* **15**, 1793-1801.

Chatterji, U., Grant , C. K. & Elder, J. H. (2000). Feline immunodeficiency virus Vif localizes to the nucleus. *Journal of Virology* **74**, 2533-2540.

Chen, D., Li, X., Zhai, Z. & Shu, H. B. (2002). A novel zinc finger protein interacts with receptor-interacting protein (RIP) and inhibits tumor necrosis factor (TNF)- and IL1-induced NF-kappa B activation. *The Journal of Biological Chemistry* **277**, 15985-15991.

Chermann, J. C., Barre-Sinoussi, F., Dauguet, C., Brun-Vezinet, F., Rouzioux, C., Rozenbaum, W. & Montagnier, L. (1983). Isolation of a new retrovirus in a patient at risk for acquired immunodeficiency syndrome. *Antibiotics & Chemotherapy* **32**, 48-53.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. & Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **85**, 1135-1148.

Chuang, T. H. & Ulevitch, R. J. (2004). Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors. *Nature Immunology* **5**, 495-502.

Chun, T. W., Stuyver, L., Mizell, S. B., Ehler, L. A., Mican, J. A., Baseler, M., Lloyd, A. L., Nowak, M. A. & Fauci, A. S. (1997). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 13193-13197.

Chung, M. Y., Lu, J. H., Weng, Y. Y. & Hwang, B. (2001). Absence of mutations in human ubiquitin fusion-degradation protein gene in tetralogy of Fallot. *Journal of Molecular Medicine* **79**, 338-342.

Coffin, J. M. (1992). Genetic diversity and evolution of retroviruses. *Current Topics In Microbiology and Immunology* **176**, 143-164.

Cooper, D. A. (1985). Acute AIDS retrovirus infection. Definition of a clinical illness associated with seroconversion. *Lancet* **1**, 537-540

Cujec, T. P., Okamoto, H., Fujinaga, K., Meyer, J., Chamberlin, H., Morgan, D. O. & Peterlin, B. M. (1997). The HIV transactivator TAT binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. *Genes & Development* **11**, 2645-2657.

Cullen, B. R. (1998). HIV-1 auxiliary proteins: making connections in a dying cell. *Cell* **93**, 685-692.

Daniel, V., Sadeghi, M., Naujokat, C., Weimer, R., Huth-Kuhne, A., Zimmermann, R. & Opelz, G. (2004). Evidence for autoantibody-induced CD4 depletion mediated by apoptotic and non-apoptotic mechanisms in HIV-positive long-term surviving haemophilia patients. *Clinical and Experimental Immunology* **135**, 94-104.

de Parseval, A., Su, S. V., Elder, J. H. & Lee, B. (2004). Specific interaction of feline immunodeficiency virus surface glycoprotein with human DC-SIGN. *Journal of Virology* **78**, 2597-2600.

Deacon, N. J., Tsykin, A., Solomon, A., Smith, K., Ludford-Menting, M., Hooker, D. J., McPhee, D. A., Greenway, A. L., Ellett, A., Chatfield, C. & et al. (1995). Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. *Science* **270**, 988-991.

Deen, K. C., McDougal, J. S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P. J., Axel, R. & Sweet, R. W. (1988). A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature* **331**, 82-84.

Demirov, D. G., Orenstein, J. M. & Freed, E. O. (2002). The late domain of human immunodeficiency virus type 1 p6 promotes virus release in a cell type-dependent manner. *Journal of Virology* **76**, 105-117.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. & Landau, N. R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381**, 661-666.

Dettenhofer, M., Cen, S., Carlson, B. A., Kleiman, L. & Yu, X. F. (2000). Association of human immunodeficiency virus type 1 Vif with RNA and its role in reverse transcription. *The Journal of Biological Chemistry* **74**, 8938-8945.

Dettenhofer, M. & Yu, X. F. (1999). Highly purified human immunodeficiency virus type 1 reveals a virtual absence of Vif in virions. *Journal of Virology* **73**, 1460-1467.

Dooher, J. E. & Lingappa, J. R. (2004). Conservation of a stepwise, energy-sensitive pathway involving HP68 for assembly of primate lentivirus capsids in cells. *Journal of Virology* **78**, 1645-1656.

Doranz, B. J., Grovit-Ferbas, K., Sharron, M. P., Mao, S. H., Goetz, M. B., Daar, E. S., Doms, R. W. & O'Brien, W. A. (1997). A small-molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *Journal of Experimental Medicine* **186**, 1395-1400.

Dragic, T., Trkola, A., Lin, S. W., Nagashima, K. A., Kajumo, F., Zhao, L., Olson, W. C., Wu, L., Mackay, C. R., Allaway, G. P., Sakmar, T. P., Moore, J. P. & Maddon, P. J. (1998). Amino-terminal substitutions in the

CCR5 coreceptor impair gp120 binding and human immunodeficiency virus type 1 entry. *Journal of Virology* **72**, 279-285.

Duh, E. J., Maury, W. J., Folks, T. M., Fauci, A. S. & Rabson, A. B. (1989). Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 5974-5978.

Englund, G., Theodore, T. S., Freed, E. O., Engleman, A. & Martin, M. A. (1995). Integration is required for productive infection of monocyte-derived macrophages by human immunodeficiency virus type 1. *Journal of Virology* **69**, 3216-3219.

Farnet, C. M. & Bushman, F. D. (1996). HIV cDNA integration: molecular biology and inhibitor development. *Aids* **10**, S3-11.

Felber, B. K., Drysdale, C. M. & Pavlakis, G. N. (1990). Feedback regulation of human immunodeficiency virus type 1 expression by the Rev protein. *Journal of Virology* **64**, 3734-3741.

Feng, Y., Broder, C. C., Kennedy, P. E. & Berger, E. A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* **272**, 872-877.

Finkel, T. H., Tudor-Williams, G., Banda, N. K., Cotton, M. F., Curiel, T., Monks, C., Baba, T. W., Ruprecht, R. M. & Kupfer, A. (1995). Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nature Medicine* **1**, 129-134.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. & Luhrmann, R. (1995a). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475-483.

Fischer, U., Huber, J., Boelens, W. C., Mattaj, I. W. & Luhrmann, R. (1995b). The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell* **82**, 475-483.

Fisher, A. G., Ensoli, B., Ivanoff, L., Chamberlain, M., Petteway, S., Ratner, L., Gallo, R. C. & Wong-Staal, F. (1987). The sor gene of HIV-1 is required for efficient virus transmission in vitro. *Science* **237**, 888-893.

Fletcher, C. V. (2003). Enfuvirtide, a new drug for HIV infection. *Lancet* **361**, 1577-1578.

Fornerod, M., Ohno, M., Yoshida, M. & Mattaj, I. W. (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-1060.

Fouchier, R. A., Simon, J. H., Jaffe, A. B. & Malim, M. H. (1996). Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of gag-, pol-, and env-encoded proteins. *Journal of Virology* **70**, 8263-8269.

Franke, E. K. & Luban, J. (1996). Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag-cyclophilin A interaction. *Virology* **222**, 279-282.

Franke, E. K., Yuan, H. E. & Luban, J. (1994). Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* **372**, 359-362.

Friedler, A., Blumenzweig, I., Baraz, L., Steinitz, M., Kotler, M. & Gilon, C. (1999). Peptides derived from HIV-1 Vif: a non-substrate based novel type of HIV-1 protease inhibitors. *Journal of Molecular Biology* **287**, 93-101.

Furtado, M. R., Callaway, D. S., Phair, J. P., Kunstman, K. J., Stanton, J. L., Macken, C. A., Perelson, A. S. & Wolinsky, S. M. (1999). Persistence of HIV-1 transcription in peripheral-blood mononuclear cells in patients receiving potent antiretroviral therapy. *The New England Journal of Medicine* **340**, 1614-1622.

Gabuzda, D. H., Lawrence, K., Langhoff, E., Terwilliger, E., Dorfman, T., Haseltine, W. A. & Sodroski, J. (1992). Role of vif in replication of human immunodeficiency virus type 1 in CD4+ T lymphocytes. *Journal of Virology* **66**, 6489-6495.

Gallay, P., Swingler, S., Song, J., Bushman, F. & Trono, D. (1995). HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83**, 569-576.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M., Shaw, G. M., Sharp, P. M. & Hahn, B. H. (1999). Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436-441.

Garcia, J. V. & D., M. A. (1992). Downregulation of cell surface CD4 by nef. *Research in Virology* **143**, 52-55.

Garcia, J. V. & Miller, A. D. (1991). Serine phosphorylation-independent downregulation of cell-surface CD4 by nef. *Nature* **350**, 508-511.

Garcia-Martinez, L. F., Mavankal, G., Neveu, J. M., Lane, W. S., Ivanov, D. & Gaynor, R. B. (1997). Purification of a Tat-associated kinase reveals a TFIIH complex that modulates HIV-1 transcription. *EMBO Journal* **16**, 2836-2850.

Garnier, L., Parent, L. J., Rovinski, B., Cao, S. X. & Wills, J. W. (1999). Identification of retroviral late domains as determinants of particle size. *Journal of Virology* **73**, 2309-2320.

Garnier, L., Ratner, L., Rovinski, B., Cao, S. X. & Wills, J. W. (1998). Particle size determinants in the human immunodeficiency virus type 1 Gag protein. *Journal of Virology* **72**, 4667-4677.

Garrett, E. D., Tiley, L. S. & Cullen, B. R. (1991). Rev activates expression of the human immunodeficiency virus type 1 vif and vpr gene products. *Journal of Virology* **65**, 1653-1657.

Geimonen, E., Fernandez, I., Gavrilovskaya, I. N. & Mackow, E. R. (2003). Tyrosine residues direct the ubiquitination and degradation of the NY-1 hantavirus G1 cytoplasmic tail. *Journal of Virology* **77**, 10760-10768.

Gelderblom, H. R., Hausmann, E. H., Ozel, M., Pauli, G. & Koch, M. A. (1987). Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* **156**, 171-176.

Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D. & De Wilde, M. (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59**, 103-112.

Goncalves, J., Jallepalli, P. & Gabuzda, D. H. (1994). Subcellular localization of the Vif protein of human immunodeficiency virus type 1. *Journal of Virology* **68**, 704-712.

Goncalves, J., Korin, Y., Zack, J. & Gabuzda, D. (1996). Role of Vif in human immunodeficiency virus type 1 reverse transcription. *Journal of Virology* **70**, 8701-8709.

Goncalves, J., Shi, B., Yang, X. & Gabuzda, D. (1995). Biological activity of human immunodeficiency virus type 1 Vif requires membrane targeting by C-terminal basic domains. *Journal of Virology* **69**, 7196-7204.

Gonda, M. A., Wong-Staal, F., Gallo, R. C., Clements, J. E., Narayan, O. & Gilden, R. V. (1985). Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. *Science* **227**, 173-177.

Gorry, P. R., Bristol, G., Zack, J. A., Ritola, K., Swanstrom, R., Birch, C. J., Bell, J. E., Bannert, N., Crawford, K., Wang, H., Schols, D., De Clercq, E., Kunstman, K., Wolinsky, S. M. & Gabuzda, D. (2001). Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity. *Journal of Virology* **75**, 10073-10089.

Gottlinger, H. G., Dorfman, T., Sodroski, J. G. & Haseltine, W. A. (1991). Effect of mutations affecting the p6 gag protein on human immunodeficiency virus particle release. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 3195-3199.

Gottlinger, H. G., Sodroski, J. G. & Haseltine, W. A. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 5781-5785.

Goudsmit, J., Debouck, C., Meloen, R. H., Smit, L., Bakker, M., Asher, D. M., Wolff, A. V., Gibbs, C. J., Jr. & Gajdusek, D. C. (1988). Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected

chimpanzees. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4478-4482.

Guy, B., Geist, M., Dott, K., Spehner, D., Kieny, M. P. & Lecocq, J. P. (1991). A specific inhibitor of cysteine proteases impairs a Vif-dependent modification of human immunodeficiency virus type 1 Env protein. *Journal of Virology* **65**, 1325-1331.

Haas, A. L. & Siepmann, T. J. (1997). Pathways of ubiquitin conjugation. *Faseb Journal* **11**, 1257-1268.

Harrich, D. & Hooker, B. (2002). Mechanistic aspects of HIV-1 reverse transcription initiation. *Reviews in Medical Virology* **12**, 31-45.

Harris, R., Ukayiofo, E.V. (1969). Rapid preparation for lymphocytes for tissue typing. *Lancet* **327**, 7615

Harris, R. S., Bishop, K. N., Sheehy, A. M., Craig, H. M., Petersen-Mahrt, S. K., Watt, I. N., Neuberger, M. S. & Malim, M. H. (2003). DNA deamination mediates innate immunity to retroviral infection. *Cell* **113**, 803-809.

Harvey, T. J., Anraku, I., Linedale, R., Harrich, D., Mackenzie, J., Suhrbier, A. & Khromykh, A. A. (2003). Kunjin virus replicon vectors for human immunodeficiency virus vaccine development. *Journal of Virology* **77**, 7796-7803.

Hassaine, G., Courcoul, M., Bessou, G., Barthalay, Y., Picard, C., Olive, D., Collette, Y., Vigne, R. & Decroly , E. (2001). The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral vif protein. *The Journal of Biological Chemistry* **276**, 16885-16893.

He, G., Ylisastigui, L. & Margolis, D. M. (2002). The regulation of HIV-1 gene expression: the emerging role of chromatin. *DNA and Cell Biology* **21**, 697-705.

Heinzinger, N. K., Bukinsky, M. I., Haggerty, S. A., Ragland, A. M., Kewalramani, V., Lee, M. A., Gendelman, H. E., Ratner, L., Stevenson, M. & Emerman, M. (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 7311-7315.

Henzler, T., Harmache, A., Herrmann, H., Spring, H., Suzan, M., Audoly, G., Panek, T. & Bosch, V. (2001). Fully functional, naturally occurring and C-terminally truncated variant human immunodeficiency virus (HIV) Vif does not bind to HIV Gag but influences intermediate filament structure. *Journal of General Virology* **82**, 561-573.

Hermida-Matsumoto, L. & Resh, M. D. (2000). Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *Journal of Virology* **74**, 8670-8679.

Herrmann, C. H., Gold, M. O. & Rice, A. P. (1996). Viral transactivators specifically target distinct cellular protein kinases that phosphorylate the RNA polymerase II C-terminal domain. *Nucleic Acids Research* **24**, 501-508.

Herrmann, C. H. & Rice, A. P. (1995). Lentivirus Tat proteins specifically associate with a cellular protein kinase, TAK, that hyperphosphorylates the carboxyl-terminal domain of the large subunit of RNA polymerase II: candidate for a Tat cofactor. *Journal of Virology* **69**, 1612-1620.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**, 389-392.

Ho, D. D., Neumann, A. U., Perelson, A. S., Chen, W., Leonard, J. M. & Markowitz, M. (1995). Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature* **373**, 123-126.

Hoglund, S., Ohagen, A., Lawrence, K. & Gabuzda, D. (1994). Role of vif during packing of the core of HIV-1. *Virology* **201**, 349-355.

Hong, H., Neamati, N., Wang, S., Nicklaus, M. C., Mazumder, A., Zhao, H., Burke, T. R., Jr., Pommier, Y. & Milne, G. W. (1997). Discovery of HIV-1 integrase inhibitors by pharmacophore searching. *Journal of Medicinal Chemistry* **40**, 930-936.

Howe, A. Y., Jung, J. U. & Desrosiers, R. C. (1998). Zeta chain of the T-cell receptor interacts with nef of simian immunodeficiency virus and human immunodeficiency virus type 2. *Journal of Virology* **72**, 9827-9834.

Huang, M. & Martin, M. A. (1997). Incorporation of Pr160(gag-pol) into virus particles requires the presence of both the major homology region and adjacent C-terminal capsid sequences within the Gag-Pol polyprotein. *Journal of Virology* **71**, 4472-4478.

Huet, T., Cheynier, R., Meyerhans, A., Roelants, G. & Wain-Hobson, S. (1990). Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* **345**, 356-359.

Hwang, S. S., Boyle, T. J., Lyerly, H. K. & Cullen, B. R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science* **253**, 71-74.

Inubushi, R. & Adachi, A. (1999). Cell-dependent function of HIV-1 Vif for virus replication. *International Journal of Molecular Medicine* **3**, 473-476.

Jacotot, E., Krust, B., Callebaut, C., Laurent-Crawford, A. G., Blanco, J. & Hovanessian, A. G. (1997). HIV-1 envelope glycoproteins-mediated apoptosis is regulated by CD4 dependent and independent mechanisms. *Apoptosis* **2**, 47-60.

Janssens, W., Fransen, K., Peeters, M., Heyndrickx, L., Motte, J., Bedjabaga, L., Delaporte, E., Piot, P. & van der Groen, G. (1994). Phylogenetic analysis of a new chimpanzee lentivirus SIVcpz-gab2 from a wild-captured chimpanzee from Gabon. *Aids Research and Human Retroviruses* **10**, 1191-1192.

Kalichman, S. C. (1998). Influencing HIV transmission risk. *Focus* **13**, 1-4.

Kao, S., Akari, H., Khan, M. A., Dettenhofer, M., Yu, X. F. & Strebel, K. (2003a). Human immunodeficiency virus type 1 Vif is efficiently packaged into virions during productive but not chronic infection. *Journal of Virology* **77**, 1131-1140.

Kao, S., Khan, M. A., Miyagi, E., Plishka, R., Buckler-White, A. & Strebel, K. (2003b). The human immunodeficiency virus type 1 Vif protein reduces intracellular expression and inhibits packaging of APOBEC3G (CEM15), a cellular inhibitor of virus infectivity. *Journal of Virology* **77**, 11398-11407.

Kao, S. Y., Calman, A. F., Luciw, P. A. & Peterlin, B. M. (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**, 489-493.

Karczewski, M. K. & Strebel, K. (1996). Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *Journal of Virology* **70**, 494-507.

Karn, J. (1999). Tackling Tat. *Journal of Molecular Biology* **293**, 235-254.

Kaul, R., Dong, T., Plummer, F. A., Kimani, J., Rostron, T., Kiama, P., Njagi, E., Irungu, E., Farah, B., Oyugi, J., Chakraborty, R., MacDonald, K. S., Bwayo, J. J., McMichael, A. & Rowland-Jones, S. L. (2001). CD8(+) lymphocytes respond to different HIV epitopes in seronegative and infected subjects. *Journal of Clinical Investigation* **107**, 1303-1310.

Kawamura, T., Qualbani, M., Thomas, E. K., Orenstein, J. M. & Blauvelt, A. (2001). Low levels of productive HIV infection in Langerhans cell-like dendritic cells differentiated in the presence of TGF-beta1 and increased viral replication with CD40 ligand-induced maturation. *European Journal of Immunology* **31**, 360-368.

Kedzierska, K. & Crowe, S. M. (2002). The role of monocytes and macrophages in the pathogenesis of HIV-1 infection. *Current Medicinal Chemistry* **9**, 1893-1903.

Kessler, M. & Mathews, M. B. (1992). Premature termination and processing of human immunodeficiency virus type 1-promoted transcripts. *Journal of Virology* **66**, 4488-4496.

Khan, M. A., Aberham, C., Kao, S., Akari, H., Gorelick, R., Bour, S. & Strebel, K. (2001). Human immunodeficiency virus type 1 Vif protein is packaged into the nucleoprotein complex through an interaction with viral genomic RNA. *Journal of Virology* **75**, 7252-7265.

Khan, M. A., Akari, H., Kao, S., Aberham, C., Davis, D., Buckler-White, A. & Strebel, K. (2002). Intravirion processing of the human immunodeficiency virus type 1 Vif protein by the viral protease may be correlated with Vif function. *Journal of Virology* **76**, 9112-9123.

Kim, S. Y., Byrn, R., Groopman, J. & Baltimore, D. (1989). Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression. *Journal of Virology* **63**, 3708-3713.

Kimpton, J. & Emerman, M. (1992). Detection of replication-competent and pseudotyped human immunodeficiency virus with a sensitive cell line on the basis of activation of an integrated beta-galactosidase gene. *Journal of Virology* **66**, 2232-2239.

Kinloch-de Loes, S., de Saussure, P., Saurat, J. H., Stalder, H., Hirschel, B. & Perrin, L. H. (1993). Symptomatic primary infection due to human immunodeficiency virus type 1: *Clinical Infectious Diseases* **17**, 59-65.

Kirchhoff, F., Greenough, T. C., Brettler, D. B., Sullivan, J. L. & Desrosiers, R. C. (1995). Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *The New England Journal of Medicine* **332**, 228-232.

Klarmann, G. J., Chen, X., North, T. W. & Preston, B. D. (2003). Incorporation of uracil into minus strand DNA affects the specificity of plus strand synthesis initiation during lentiviral reverse transcription. *Journal of Biological Chemistry* **278**, 7902-7909.

Klimkait, T., Strebel, K., Hoggan, M. D., Martin, M. A. & Orenstein, J. M. (1990). The human immunodeficiency virus type 1-specific protein vpu is

required for efficient virus maturation and release. *Journal of Virology* **64**, 621-629.

Kobor, M. S. & Greenblatt, J. (2002). Regulation of transcription elongation by phosphorylation. *Biochimica et Biophysica Acta* **1577**, 261-275.

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A., Scolnick, E. M. & Sigal, I. S. (1988). Active human immunodeficiency virus protease is required for viral infectivity. *Proceedings of the National Academy of Sciences of the United States of America* **85**, 4686-4690.

Korber, B., Muldoon, M., Theiler, J., Gao, F., Gupta, R., Lapedes, A., Hahn, B. H., Wolinsky, S. & Bhattacharya, T. (2000). Timing the ancestor of the HIV-1 pandemic strains. *Science* **288**, 1789-1796.

Kumar, S., Orsini, M. J., Lee, J. C., McDonnell, P. C., Debouck, C. & Young, P. R. (1996). Activation of the HIV-1 long terminal repeat by cytokines and environmental stress requires an active CSBP/p38 MAP kinase. *The Journal of Biological Chemistry* **271**, 30864-30869.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393**, 648-659.

Kwong, P. D., Wyatt, R., Sattentau, Q. J., Sodroski, J. & Hendrickson, W. A. (2000). Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. *Journal of Virology* **74**, 1961-1972.

Lake, J. A., Carr, J., Feng, F., Mundy, L., Burrell, C. & Li, P. (2003). The role of Vif during HIV-1 infection: interaction with novel host cellular factors. *Journal of Clinical Virology* **26**, 143-152.

Landau, N. R., Warton, M. & Littman, D. R. (1988). The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4. *Nature* **334**, 159-162.

Lapadat-Tapolsky, M., De Rocquigny, H., Van Gent, D., Roques, B., Plasterk, R. & Darlix, J. L. (1993). Interactions between HIV-1 nucleocapsid protein and viral DNA may have important functions in the viral life cycle.[erratum appears in Nucleic Acids Res 1993 Apr 25;21(8):2024]. *Nucleic Acids Research* **21**, 831-839.

Laspia, M. F., Rice, A. P. & Mathews, M. B. (1989a). HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59**, 283-292.

Laspia, M. F., Rice, A. P. & Mathews, M. B. (1989b). HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. *Cell* **59**, 283-292.

Lecossier, D., Bouchonnet, F., Clavel, F. & Hance, A. J. (2003). Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* **300**, 1112.

Li, P., Stephenson, A. J., Kuiper, L. J. & Burrell, C. J. (1993). Double-stranded strong-stop DNA and the second template switch in human immunodeficiency virus (HIV) DNA synthesis. *Virology* **194**, 82-88.

Lichtner, M., Maranon, C., Vidalain, P. O., Azocar, O., Hanau, D., Lebon, P., Burgard, M., Rouzioux, C., Vullo, V., Yagita, H., Rabourdin-Combe, C., Servet, C. & Hosmalin, A. (2004). HIV type 1-infected dendritic cells induce apoptotic death in infected and uninfected primary CD4 T lymphocytes. *Aids Research and Human Retroviruses* **20**, 175-182.

Lim, M. L., Dong, B. J. & Hart, S. L. (2003). Outcomes of dual-protease inhibitor salvage therapy in human immunodeficiency virus-infected patients referred to a telephone consultation service. *Pharmacotherapy* **23**, 1100-1104.

Liu, H., Wu, X., Newman, M., Shaw, G. M., Hahn, B. H. & Kappes, J. C. (1995a). The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *Journal of Virology* **69**, 7630-7638.

Liu, H., Wu, X., Newman, M., Shaw, G. M., Hahn, B. H. & Kappes, J. C. (1995b). The Vif protein of human and simian immunodeficiency viruses is packaged into virions and associates with viral core structures. *Journal of Virology* **69**, 7630-7638.

Lore, K. & Larsson, M. (2003). The role of dendritic cells in the pathogenesis of HIV-1 infection. *Apmis* **111**, 776-788.

Luban, J. (1996). Absconding with the chaperone: essential cyclophilin-Gag interaction in HIV-1 virions. *Cell* **87**, 1157-1159.

Lundquist, C. A., Tobiume, M., Zhou, J., Unutmaz, D. & Aiken, C. (2002). Nef-mediated downregulation of CD4 enhances human immunodeficiency virus type 1 replication in primary T lymphocytes. *Journal of Virology* **76**, 4625-4633.

Luria, S., Chambers, I. & Berg, P. (1991). Expression of the type 1 human immunodeficiency virus Nef protein in T cells prevents antigen receptor-mediated induction of interleukin 2 mRNA. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 5326-5330.

Madani, N. & Kabat, D. (1998). An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *Journal of Virology* **72**, 10251-10255.

Madani, N., Millette, R., Platt, E. J., Marin, M., Kozak, S. L., Bloch, D. B. & Kabat, D. (2002). Implication of the lymphocyte-specific nuclear body protein Sp140 in an innate response to human immunodeficiency virus type 1. *Journal of Virology* **76**, 11133-11138.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G. D., Muschel, R. J. & Weiner, D. B. (1998). HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G2/M phase transition of the mammalian cell cycle. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 3419-3424.

Malim, M. H. & Cullen, B. R. (1991). HIV-1 structural gene expression requires the binding of multiple Rev monomers to the viral RRE: implications for HIV-1 latency. *Cell* **65**, 241-248.

Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L. & Trono, D. (2003). Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**, 99-103.

Mariani, R., Chen, D., Schrofelbauer, B., Navarro, F., Konig, R., Bollman, B., Munk, C., Nymark-McMahon, H. & Landau, N. R. (2003). Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* **114**, 21-31.

Marin, M., Rose, K. M., Kozak, S. L. & Kabat, D. (2003). HIV-1 Vif protein binds the editing enzyme APOBEC3G and induces its degradation. *Nature Medicine* **9**, 1398-1403.

Marshall, W. L., Diamond, D. C., Kowalski, M. M. & Finberg, R. W. (1992). High level of surface CD4 prevents stable human immunodeficiency virus infection of T-cell transfectants. *Journal of Virology* **66**, 5492-5499.

McMichael, A. J. & Hanke, T. (2003). HIV vaccines 1983-2003. *Nature Medicine* **9**, 874-880.

Mellors, J. W., Rinaldo, C. R., Jr., Gupta, P., White, R. M., Todd, J. A. & Kingsley, L. A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. *Science* **272**, 1167-1170.

Meyaard, L., Otto, S. A., Jonker, R. R., Mijnster, M. J., Keet, R. P. & Miedema, F. (1992). Programmed death of T cells in HIV-1 infection. *Science* **257**, 217-219.

Migueles, S. A. & Connors, M. (2002). The Role of CD4(+) and CD8(+) T Cells in Controlling HIV Infection. *Current Infectous Disease Reports* **4**, 461-467.

Miller, M. D., Warmerdam, M. T., Gaston, I., Greene, W. C. & Feinberg, M. B. (1994). The human immunodeficiency virus-1 nef gene product: a positive factor for viral infection and replication in primary lymphocytes and macrophages. *Journal of Experimental Medicine* **179**, 101-113.

Mitsuya, H. & Broder, S. (1987). Strategies for antiviral therapy in AIDS. *Nature* **325**, 773-778.

Mondor, I., Ugolini, S. & Sattentau, Q. J. (1998). Human immunodeficiency virus type 1 attachment to HeLa CD4 cells is CD4 independent and gp120 dependent and requires cell surface. heparans. *Journal of Virology* **72**, 3623-3634.

Moynihan, T. P., Ardley, H. C., Nuber, U., Rose, S. A., Jones, P. F., Markham, A. F., Scheffner, M. & Robinson, P. A. (1999). The ubiquitin-conjugating

enzymes UbcH7 and UbcH8 interact with RING finger/IBR motif-containing domains of HHARI and H7-AP1. *The Journal of Biological Chemistry* **274**, 30963-30968.

Nabel, G. & Baltimore, D. (1987). An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* **326**, 711-713.

Niu, M. T., Stein, D. S. & Schnittman, S. M. (1993). Primary human immunodeficiency virus type 1 infection: review of pathogenesis and early treatment intervention in humans and animal retrovirus infections. *Journal of Infectious Diseases* **168**, 1490-1501.

Oberste, M. S. & Gonda, M. A. (1992). Conservation of amino-acid sequence motifs in lentivirus Vif proteins. *Virus Genes* **6**, 95-102.

O'Brien, W. A. (2003). New classes of HIV drugs on the horizon. A review of the presentation at the satellite symposium "New hope: advancing care in HIV infection" at the 15th annual Association of Nurses in AIDS Care conference, November 2002. *AIDS Reader* **13**, S4-8.

Ohagen, A. & Gabuzda, D. (2000). Role of Vif in stability of the human immunodeficiency virus type 1 core. *Journal of Virology* **74**, 11055-11066.

Pandori, M. W., Fitch, N. J., Craig, H. M., Richman, D. D., Spina, C. A. & Guatelli, J. C. (1996). Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. *Journal of Virology* **70**, 4283-4290.

Parkin, N. T., Chamorro, M. & Varmus, H. E. (1992). Human immunodeficiency virus type 1 gag-pol frameshifting is dependent on downstream mRNA

secondary structure: demonstration by expression in vivo. *Journal of Virology* **66**, 5147-5151.

Paul, M. & Jabbar, M. A. (1997). Phosphorylation of both phosphoacceptor sites in the HIV-1 Vpu cytoplasmic domain is essential for Vpu-mediated ER degradation of CD4. *Virology* **232**, 207-216.

Paxton, W., Connor, R. I. & Landau, N. R. (1993). Incorporation of Vpr into human immunodeficiency virus type 1 virions: requirement for the p6 region of gag and mutational analysis. *Journal of Virology* **67**, 7229-7237.

Peng, C., Ho, B. K., Chang, T. W. & Chang, N. T. (1989). Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. *Journal of Virology* **63**, 2550-2556.

Perelson, A. S., Essunger, P., Cao, Y., Vesanen, M., Hurley, A., Saksela, K., Markowitz, M. & Ho, D. D. (1997). Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* **387**, 188-191.

Peterson, P. K., Gekker, G., Hu, S., Cabral, G. & Lokensgard, J. R. (2004). Cannabinoids and morphine differentially affect HIV-1 expression in CD4(+) lymphocyte and microglial cell cultures. *Journal of Neuroimmunology* **147**, 123-126.

Piguet, V. & Blauvelt, A. (2002). Essential roles for dendritic cells in the pathogenesis and potential treatment of HIV disease. *Journal of Investigative Dermatology* **119**, 365-369.

Pleskoff, O., Sol, N., Labrosse, B. & Alizon, M. (1997). Human immunodeficiency virus strains differ in their ability to infect CD4+ cells expressing the rat homolog of CXCR-4 (fusin). *Journal of Virology* **71**, 3259-3262.

Pollard, V. W. & Malim, M. H. (1998). The HIV-1 Rev protein. *Annual Review of Microbiology* **52**, 491-532.

Purcell, D. F. & Martin, M. A. (1993). Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. *Journal of Virology* **67**, 6365-6378.

Ratnasabapathy, R., Sheldon, M., Johal, L. & Hernandez, N. (1990). The HIV-1 long terminal repeat contains an unusual element that induces the synthesis of short RNAs from various mRNA and snRNA promoters. *Genes & Development* **4**, 2061-2074.

Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K. & et al. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313**, 277-284.

Rey-Cuille, M. A., Berthier, J. L., Bomsel-Demontoy, M. C., Chaduc, Y., Montagnier, L., Hovanessian, A. G. & Chakrabarti, L. A. (1998). Simian immunodeficiency virus replicates to high levels in sooty mangabeys without inducing disease. *Journal of Virology* **72**, 3872-3886.

Rice, A. P. & Mathews, M. B. (1988). Transcriptional but not translational regulation of HIV-1 by the tat gene product. *Nature* **332**, 551-553.

Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A. & Walker, B. D. (1997). Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* **278**, 1447-1450.

Rowland-Jones, S. L., Dong, T., Fowke, K. R., Kimani, J., Krausa, P., Newell, H., Blanchard, T., Ariyoshi, K., Oyugi, J., Ngugi, E., Bwayo, J., MacDonald, K. S., McMichael, A. J. & Plummer, F. A. (1998). Cytotoxic

T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. *Journal of Clinical Investigation* **102**, 1758-1765.

Rucker, J., Samson, M., Doranz, B. J., Libert, F., Berson, J. F., Yi, Y., Smyth, R. J., Collman, R. G., Broder, C. C., Vassart, G., Doms, R. W. & Parmentier, M. (1996). Regions in beta-chemokine receptors CCR5 and CCR2b that determine HIV-1 cofactor specificity. *Cell* **87**, 437-446.

Saha, K., Zhang, J., Gupta, A., Dave, R., Yimen, M. & Zerhouni, B. (2001). Isolation of primary HIV-1 that target CD8+ T lymphocytes using CD8 as a receptor. *Nature Medecine* **7**, 65-72.

Sakai, H., Kawamura, M., Sakuragi, J., Sakuragi, S., Shibata, R., Ishimoto, A., Ono, N., Ueda, S. & Adachi, A. (1993a). Integration is essential for efficient gene expression of human immunodeficiency virus type 1. *Journal of Virology* **67**, 1169-1174.

Sakai, H., Shibata, R., Sakuragi, J., Sakuragi, S., Kawamura, M. & Adachi, A. (1993b). Cell-dependent requirement of human immunodeficiency virus type 1 Vif protein for maturation of virus particles. *Journal of Virology* **67**, 1663-1666.

Sandri-Goldin, R. M. (2004). MINIREVIEW:Viral Regulation of mRNA Export. *Journal of Virology*, 4389-4396.

Schacker, T., Collier, A. C., Hughes, J., Shea, T. & Corey, L. (1996). Clinical and epidemiologic features of primary HIV infection. *Annals of Internal Medicine* **125**, 257-264.

Schwartz, O., Dautry-Varsat, A., Goud, B., Marechal, V., Subtil, A., Heard, J. M. & Danos, O. (1995a). Human immunodeficiency virus type 1 Nef

induces accumulation of CD4 in early endosomes. *Journal of Virology* **69**, 528-533.

Schwartz, O., Marechal, V., Danos, O. & Heard, J. M. (1995b). Human immunodeficiency virus type 1 Nef increases the efficiency of reverse transcription in the infected cell. *Journal of Virology* **69**, 4053-4059.

Schwartz, S., Felber, B. K., Fenyo, E. M. & Pavlakis, G. N. (1990). Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. *Journal of Virology* **64**, 5448-5456.

Schwartz, S., Felber, B. K. & Pavlakis, G. N. (1991). Expression of human immunodeficiency virus type 1 vif and vpr mRNAs is Rev-dependent and regulated by splicing. *Virology* **183**, 677-686.

Sheehy, A. M., Gaddis, N. C., Choi, J. D. & Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**, 646-650.

Sheehy, A. M., Gaddis, N. C. & Malim, M. H. (2003). The antiretroviral enzyme APOBEC3G is degraded by the proteasome in response to HIV-1 Vif. *Nature Medicine* **9**, 1404-1407.

Shimura, H., Schlossmacher, M. G., Hattori, N., Frosch, M. P., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, K. S. & Selkoe, D. J. (2001). Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. *Science* **293**, 263-269.

Shiraishi, T., Misumi, S., Takama, M., Takahashi, I. & Shoji, S. (2001). Myristoylation of human immunodeficiency virus type 1 gag protein is required for efficient env protein transportation to the surface of cells. *Biochemical and Biophysical Research Communications* **282**, 1201-1205.

Shiver, J. W., Fu, T. M., Chen, L., Casimiro, D. R., Davies, M. E., Evans, R. K., Zhang, Z. Q., Simon, A. J., Trigona, W. L., Dubey, S. A., Huang, L., Harris, V. A., Long, R. S., Liang, X., Handt, L., Schleif, W. A., Zhu, L., Freed, D. C., Persaud, N. V., Guan, L., Punt, K. S., Tang, A., Chen, M., Wilson, K. A., Collins, K. B., Heidecker, G. J., Fernandez, V. R., Perry, H. C., Joyce, J. G., Grimm, K. M., Cook, J. C., Keller, P. M., Kresock, D. S., Mach, H., Troutman, R. D., Isopi, L. A., Williams, D. M., Xu, Z., Bohannon, K. E., Volkin, D. B., Montefiori, D. C., Miura, A., Krivulka, G. R., Lifton, M. A., Kuroda, M. J., Schmitz, J. E., Letvin, N. L., Caulfield, M. J., Bett, A. J., Youil, R., Kaslow, D. C. & Emini, E. A. (2002). Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. *Nature* **415**, 331-335.

Silvestri, G., Sodora, D. L., Koup, R. A., Paiardini, M., O'Neil, S. P., McClure, H. M., Staprans, S. I. & Feinberg, M. B. (2003). Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. *Immunity* **18**, 441-452.

Simon, J. H., Carpenter, E. A., Fouchier, R. A. & Malim, M. H. (1999a). Vif and the p55(Gag) polyprotein of human immunodeficiency virus type 1 are present in colocalizing membrane-free cytoplasmic complexes. *Journal of Virology* **73**, 2667-2674.

Simon, J. H., Fouchier, R. A., Southerling, T. E., Guerra, C. B., Grant, C. K. & Malim, M. H. (1997). The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. *Journal of Virology* **71**, 5259-5267.

Simon, J. H., Gaddis, N. C., Fouchier, R. A. & Malim, M. H. (1998a). Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nature Medicine* **4**, 1397-1400.

Simon, J. H. & Malim, M. H. (1996). The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. *Journal of Virology* **70**, 5297-5305.

Simon, J. H., Miller, D. L., Fouchier, R. A., Soares, M. A., Peden, K. W. & Malim, M. H. (1998b). The regulation of primate immunodeficiency virus infectivity by Vif is cell species restricted: a role for Vif in determining virus host range and cross-species transmission. *EMBO Journal* **17**, 1259-1267.

Simon, J. H., Sheehy, A. M., Carpenter, E. A., Fouchier, R. A. & Malim, M. H. (1999b). Mutational analysis of the human immunodeficiency virus type 1 Vif protein. *Journal of Virology* **73**, 2675-2681.

Singer, A. L., Bunnell S, C., Obstfeld, A. E., Jordan, M. S., Wu, J. N., Myung, P. S., samelson, L. I. & Koretzky, G. A. (2004). Role of the proline-Rich Domain in SLP-76 Subcellular Localization and T Cell Function. *The Journal of Biological Chemistry* **279** (15), 15481–15490.

Skowronski, J., Parks, D. & Mariani, R. (1993). Altered T cell activation and development in transgenic mice expressing the HIV-1 nef gene. *EMBO Journal* **12**, 703-713.

Smith, D. H., Byrn, R. A., Marsters, S. A., Gregory, T., Groopman, J. E. & Capon, D. J. (1987). Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen. *Science* **238**, 1704-1707.

Soll, D. R. (1997). Researchers in cell motility and the cytoskeleton can play major roles in understanding AIDS. *Cell Motility & the Cytoskeleton* **37**, 91-97.

Sova, P. & Volsky, D. J. (1993). Efficiency of viral DNA synthesis during infection of permissive and nonpermissive cells with vif-negative human immunodeficiency virus type 1. *Journal of Virology* **67**, 6322-6326.

Sova, P., Volsky, D. J., Wang, L. & Chao, W. (2001). Vif is largely absent from human immunodeficiency virus type 1 mature virions and associates mainly with viral particles containing unprocessed gag. *Journal of Virology* **75**, 5504-5517.

Srinivasakumar, N., Hammarskjold, M. L. & D, R. (1995). Characterization of deletion mutations in the capsid region of human immunodeficiency virus type 1 that affect particle formation and Gag-Pol precursor incorporation. *Journal of Virology* **69**, 6106-6114.

Stade, K., Ford, C. S., Guthrie, C. & Weis, K. (1997). Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-1050.

Stevenson, M., Stanwick, T. L., Dempsey, M. P. & Lamonica, C. A. (1990). HIV-1 replication is controlled at the level of T cell activation and proviral integration. *EMBO Journal* **9**, 1551-1560.

Stoddart, C. A., Geleziunas, R., Ferrell, S., Linquist-Stepps, V., Moreno, M. E., Bare, C., Xu, W., Yonemoto, W., Bresnahan, P. A., McCune, J. M. & Greene, W. C. (2003). Human immunodeficiency virus type 1 Nef-mediated downregulation of CD4 correlates with Nef enhancement of viral pathogenesis. *Journal of Virology* **77**, 2124-2133.

Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T. & Martin, M. A. (1987). The HIV 'A' (sor) gene product is essential for virus infectivity. *Nature* **328**, 728-730.

Swingler, S., Brichacek, B., Jacque, J. M., Ulich, C., Zhou, J. & Stevenson, M. (2003). HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection. *Nature* **424**, 213-219.

Terwilliger, E. F., Cohen, E. A., Lu, Y. C., Sodroski, J. G. & Haseltine, W. A. (1989). Functional role of human immunodeficiency virus type 1 vpu *Proceedings of the National Academy of Sciences of the United States of America* **86**, 5163-5167.

Thorsby, E. Bratlie, A. (1970). A rapid method for preparation of pure lymphocyte suspensions. *Histocompatibility Testing 1970*, 655

Thunnissen, E. B., Baak, J. P., Diegenbach, P. C. & Velthuis, P. J. (1981). Fixation induced variations in quantitative nuclear image features in sections. *Acta Histochemica* **68**, 218-226.

Tong-Starksen, S. E., Luciw, P. A. & Peterlin, B. M. (1987). Human immunodeficiency virus long terminal repeat responds to T-cell activation signals. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 6845-6849.

Toohey, M. G. & Jones, K. A. (1989). In vitro formation of short RNA polymerase II transcripts that terminate within the HIV-1 and HIV-2 promoter-proximal downstream regions. *Genes & Development* **3**, 265-282.

Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., Cheng-Mayer, C., Robinson, J., Maddon, P. J. & Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature* **384**, 184-187.

Trono, D. (1995). HIV accessory proteins: leading roles for the supporting cast. *Cell* **82**, 189-192.

Ulich, C., Dunne, A., Parry, E., Hooker, C. W., Gaynor, R. B. & Harrich, D. (1999). Functional domains of Tat required for efficient human immunodeficiency virus type 1 reverse transcription. *Journal of Virology* **73**, 2499-2508.

Unutmaz, D., KewalRamani, V. N., Marmon, S. & Littman, D. R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *Journal of Experimental Medicine* **189**, 1735-1746.

Valerie, K., Delers, A., Bruck, C., Thiriart, C., Rosenberg, H., Debouck, C. & Rosenberg, M. (1988). Activation of human immunodeficiency virus type 1 by DNA damage in human cells. *Nature* **333**, 78-81.

van der Reijden, B. A., Erpelinck-Verschueren, C. A., Lowenberg, B. & Jansen, J. H. (1999). TRIADs: a new class of proteins with a novel cysteine-rich signature. *Protein Science* **8**, 1557-1561.

Van Lint, C., Emiliani, S., Ott, M. & Verdin, E. (1996). Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO Journal* **15**, 1112-1120.

Vandegraaff, N., Kumar, R., Hocking, H., R., T., JR., B., Mills, J., Rhodes, D., Burrell, C. J. & Li, P. (2001). Specific inhibition of human immunodeficiency virus type 1 (HIV-1) integration in cell culture: putative inhibitors of HIV-1 integrase. *Antimicrobial Agents & Chemotherapy* **45**, 2510-2516.

Vanden Haesevelde, M. M., Peeters, M., Jannes, G., Janssens, W., van der Groen, G., Sharp, P. M. & Saman, E. (1996). Sequence analysis of a highly divergent HIV-1-related lentivirus isolated from a wild captured chimpanzee. *Virology* **221**, 346-350.

Volsky, D. J., Potash, M. J., Simm, M., Sova, P., Ma, X. Y., Chao, W. & Shahabuddin, M. (1995). The human immunodeficiency virus type 1 vif gene: the road from an accessory to an essential role in human immunodeficiency virus type 1 replication. *Current Topics in Microbiology & Immunology* **193**, 157-168.

von Schwedler, U., Song, J., Aiken, C. & Trono, D. (1993). Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *Journal of Virology* **67**, 4945-4955.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S. & Alizon, M. (1985). Nucleotide sequence of the AIDS virus, LAV. *Cell* **40**, 9-17.

Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H. & Jones, K. A. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**, 451-462.

Wei, X., Ghosh, S. K., Taylor, M. E., Johnson, V. A., Emini, E. A., Deutsch, P., Lifson, J. D., Bonhoeffer, S., Nowak, M. A., Hahn, B. H. & et al. (1995). Viral dynamics in human immunodeficiency virus type 1 infection. *Nature* **373**, 117-122.

Weiss, R. A. (1993). How does HIV cause AIDS? *Science* **260**, 1273-1279.

Weiss, R. A., Clapham, P. R., McClure, M. O., McKeating, J. A., McKnight, A., Dalgleish, A. G., Sattentau, Q. J. & Weber, J. N. (1988). Human immunodeficiency viruses: neutralization and receptors. *Journal of Acquired Immune Deficiency Syndrome* **1**, 536-541.

Wills, J. W. & Craven, R. C. (1991). Form, function, and use of retroviral gag proteins. *Aids* **5**, 639-654.

Wu, L., Gerard, N. P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A. A., Desjardin, E., Newman, W., Gerard, C. & Sodroski, J. (1996). CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* **384**, 179-183.

Xiao, X., Kinter, A., Broder, C. C. & Dimitrov, D. S. (2000). Interactions of CCR5 and CXCR4 with CD4 and gp120 in human blood monocyte-derived dendritic cells. *Experimental and Molecular Pathology* **68**, 133-138.

Yang, S., Sun, Y. & Zhang, H. (2001). The multimerization of human immunodeficiency virus type I Vif protein: a requirement for Vif function in the viral life cycle. *The Journal of Biological Chemistry* **276**, 4889-4893.

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P. & Yu, X. F. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**, 1056-1060.

Zhang, C., Rasmussen, C. & Chang, L. J. (1997). Cell cycle inhibitory effects of HIV and SIV Vpr and Vpx in the yeast Schizosaccharomyces pombe. *Virology* **230**, 103-112.

Zhang, H., Pomerantz, R. J., Dornadula, G. & Sun, Y. (2000a). Human immunodeficiency virus type 1 Vif protein is an integral component of an mRNP complex of viral RNA and could be involved in the viral RNA folding and packaging process. *Journal of Virology* **74**, 8252-8261.

Zhang, H., Yang, B., Pomerantz, R. J., Zhang, C., Arunachalam, S. C. & Gao, L. (2003). The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**, 94-98.

Zhang, Y., Gao, J., Chung, K. K., Huang, H., Dawson, V. L. & Dawson, T. M. (2000b). Parkin functions as an E2-dependent ubiquitin- protein ligase and

promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13354-13359.

Ziegler, J. B. (1990). Prenatal diagnoses of HIV infection. *Journal of Acquired Immune Deficiency Syndrome* **3**, 548.

Zimmerman, C., Klein, K. C., Kiser, P. K., Singh, A. R., Firestein, B. L., Riba, S. C. & Lingappa, J. R. (2002). Identification of a host protein essential for assembly of immature HIV-1 capsids. *Nature* **415**, 88-92.